Assessment of mosquito larvicide impacts on aquatic invertebrates in the Vasse-Wonnerup Wetland System 2009 - 2015

Report to the City of Busselton

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Cover:

Birds of the Vasse-Wonnerup wetlands. Top, left to right are some of the birds shown that are important for Ramsar listing of the Vasse-Wonnerup, through supporting more than 1% of the total population: the Red-necked avocet (waders.org.au), the Black-winged stilt (naturephoto-cz.com) and the Australian shelduck (ozanimals.com). Black swans below (R. Paice).

Summary

The City of Busselton undertakes seasonal mosquito larvicide treatment at several breeding sites, including areas of the Vasse-Wonnerup wetland system. Due to the significance of these wetlands as waterbird habitat, monitoring of non-target aquatic invertebrates is required to ensure that food resources for waterbirds are not negatively impacted by the use of larvicide. A sampling program to fulfil this requirement was developed in 2009 and has been undertaken consistently in each season since then. This report presents the long term results of this sampling program, from 2009 to 2015, which include pre-and post-treatment sampling in three treatment and three control wetlands associated with 19 applications of S-Methoprene and 3 applications of *Bacillus thuringiensis* var. *israelensis* (*Bti*). A summary of larvicide efficacy, based on mosquito larvae sampling undertaken by the City of Busselton, is also presented.

Aquatic invertebrate sampling over seven years has found no evidence of adverse impacts of larvicide use on non-target organisms, for either S-Methoprene or *Bti*. The indicator groups Ostracoda and Amphipoda were the most abundant macroinvertebrate taxa, along with Chironomidae. Larvicide treatments had no negative effects on abundance of these groups, nor was there any impact found for other macroinvertebrate groups, zooplankton or aquatic invertebrate diversity. Community composition was similar in treatment and control sites. Variation in abundance of invertebrates, taxa richness and community assemblage in samples was strongly dependent on application date, indicating that natural variation over time affects these samples rather than any effect of larvicide application.

Larvicide emergence testing indicated that application of the S-methoprene hormonal larvicide was effective in preventing a high percentage (mean = 84%) of adult mosquitos from emerging. Pre- and post-treatment sampling at treated sites indicated high efficacy of Bti, with mean reduction in larvae abundance of 95% for the three applications in 2014 and 2015.

The overall aim of non-target invertebrate sampling is to assess any indirect impacts on waterbirds through reduced availability of aquatic invertebrates as a food resource is therefore highly unlikely. A lack of any negative effects shown in this study corresponds with other research throughout the world, which also suggests that these control agents are not harmful to non-target organisms at the application rates required for mosquito control. For S-Methoprene, these results are considered conclusive owing to the long term data set. For *Bti*, only three applications have been undertaken and ongoing sampling associated with its use would result in a more robust assessment.

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1 Introduction

The City of Busselton is located about 200km south of Perth, Western Australia. Much of the residential development in the City has occurred on areas of the Swan Coastal Plain in close proximity to floodplain and wetland areas. As these areas include habitat favourable to mosquito breeding, mosquitoes are sometimes a problem to residents both as a nuisance pest and as vectors of disease.

To minimise the incidence of Ross River Virus amongst residents and visitors, mosquito larvicide is used in breeding sites on a seasonal basis (spring and summer) by the City of Busselton. The treatment program includes use of the hormonal agent S-methoprene and the bacterial agent *Bacillus thuringiensis* var. *israelensis* (*Bti*). Treatment is driven by external environmental factors and larval numbers ascertained through surveys. Adulticide fogging (using a pyrethroid-based agent) is included in the mosquito control program for use in residential areas in a prescribed manner when there is a high risk of Ross River Virus and a high level of complaints from residents. A comprehensive review of potential impacts of the mosquito control program is provided in an Environmental Management Plan (EMP) (Paice, 2011a).

The Vasse-Wonnerup wetland system requires treatment, and is listed as a Wetland of International Importance under the Ramsar Convention due to its significance as waterbird habitat. The wetlands regularly support more than 30,000 waterbirds annually and 78 species have been recorded (Lane et al, 2009; Australian Wetlands Database, 2010). Many species of waterbirds are dependent on aquatic invertebrates as all or part of their diet, and therefore any adverse impacts of larvicide on non-target invertebrates may indirectly affect waterbirds. The Vasse-Wonnerup wetland system is protected through the Environment Protection and Biodiversity Conservation Act 1999. Approval for mosquito control in the wetlands was given by the former Federal Department of Environment, subject to ongoing sampling to assess the effects of larvicide on non-target invertebrates. Approval also restricts the use of larvicide to a maximum of seven aerial applications of larvicide per calendar year, supplemented by hand treatment of small areas.

To identify any adverse impacts on non-target aquatic invertebrates, and thereby potential impact on waterbird food resources, the pre- and post-treatment sampling is undertaken for each larvicide application. This sampling program has been undertaken during each mosquito breeding season since spring 2009. During the 2009 season, a significant effect of larvicide use on abundance of *Mytilocypris* ostracods was reported (Paice 2010). However, subsequent annual reporting has not identified any negative effects on this genus or other components of the aquatic invertebrate community (see Paice 2011b, 2012, 2013, 2014, 2015, 2016), and sampling during 2012 indicated higher *Mytilocypris* abundance in treatment sites compared to controls. This report presents the long

term results of this sampling program, from 2009 to 2015, which include pre-and post-treatment sampling in three treatment and three control wetlands associated with 19 applications of S-Methoprene and 3 applications of *Bacillus thuringiensis* var. *israelensis* (*Bti*). A summary of larvicide efficacy, based on mosquito larvae sampling undertaken by the City of Busselton, is also presented.

Figure 1. Location of treatment and control wetlands included in the monitoring program. The yellow dashed line marks the Port Geographe area where pyrethroid fogging was undertaken in October 2013.

2 Methods

In accordance with DEHW approval and the EMP, sampling for this project includes aquatic invertebrate diversity and abundance at three treatment sites and three control sites, with an emphasis on indicator taxa: Amphipoda and Ostracoda. Sampling sites and methods were consistent in all mosquito control seasons throughout the period 2009-2015. For each application of larvicide, aquatic invertebrates were sampled in three treated wetlands and three untreated (control) wetlands within 48 hours prior to treatment and 24-48 hours after treatment.

The timing of larvicide treatments is determined by monitoring of mosquito breeding, undertaken by City officers on a regular basis. Analysis of numbers of mosquito larvae per square metre is used to determine the need for larvicide application, with the threshold for consideration of treatment being 100 larvae per square metre.

The number of treatments, and therefore samples, was determined by the need for mosquito control. A maximum of four larvicide applications was undertaken in any year. S-Methoprene was used predominantly, with a total of 19 aerial applications and one hand treatment (January 2012) over the seven-year period. The use of *Bti* only commenced recently, with two treatments in 2014 and one in 2015. Fogging with adulticide was conducted in the suburb of Port Geographe on 28 October 2013. Table 1 provides a summary of treatment dates and associated sampling.

Table 1. Sample dates for larvicide treatment, non-target invertebrate sampling and efficacy sampling during 2009 – 2015 mosquito control periods. S-M = S-methoprene; Bti = Bacillus thuringiensis var. israelensis; nr = date not recorded; dash indicates no data.

Year	Treatment dates	Larvicide	Pre-treatment	Post-treatment	Efficacy post-treatment
2009	19-09-09	S-M	17-09-09	20-09-09	22-09-09
	30-10-09	S-M	30-10-09	1-11-09	3-11-09
	19-11-09	S-M	19-11-09	21-11-09	24-11-09
2010	06-08-10	S-M	6-08-10	8-08-10	9-08-10
	08-10-10	S-M	7-10-10	11-10-10	11-10-10
	29-10-10	S-M	26-10-10	31-10-10	1-11-10
	18-11-10	S-M	17-11-10	20-11-10	nr
2011-12	30-09-11	S-M	29-09-11	02-10-11	5-10-11
	30-10-11	S-M	30-10-11	02-11-10	-
	20-11-11	S-M	19-11-11	21-11-11	23-11-11
	20-12-11	S-M	20-12-11	21-12-11	-
	09-01-12	S-M - hand	9-01-12	11-01-12	-
2012	09-10-12	S-M	6-10-12	11-10-12	-
	12-11-12	S-M	10-11-12	14-11-12	14-11-12
2013	23-08-13	S-M	20-08-13	24-08-13	26-08-13
	13-10-13	S-M	11-10-13	16-10-13	16-10-13
	26-10-13	S-M	24-10-13	29-10-13	29-10-13
	28-10-13	fogging			-
	18-12-13	S-M	17-12-13	20-12-13	20-12-13
2014	19-09-14	*Bti*			22-09-14
	31-10-2014	S-M	30-10-14	1-11-14	5-11-14
	04-12-2014	*Bti*			8-12-14
2015	10-10-2015	*Bti*			14-10-15
	22-10-2015	S-M	21-10-15	24-10-15	-

2.1 Site description

Vasse-Wonnerup wetland system

The Vasse-Wonnerup wetland system covers about 1000 hectares and consists of large areas of relatively shallow open waters surrounded by large areas of seasonally inundated samphire floodplain, and some areas of sedgelands and Melaleuca woodland (Bamford and Bamford, 1995). Large areas of the floodplain have been cleared and are grazed. There are two main open water wetland areas: the Vasse Estuary to the south-east and the Wonnerup Estuary to the north, as well as a smaller wetland area known as Malbup Creek, and several relatively small adjacent wetland pools.

Floodgates situated at the entrances to both the Vasse and Wonnerup Estuaries since 1908 have significantly altered the hydrological regime of the wetlands by excluding tidal exchange of seawater. New floodgates have been built and are managed during summer to allow tidal waters into the estuaries with consideration to maintaining appropriate water levels and water quality and to allow fish passage.

The Ramsar status of the Vasse-Wonnerup system is based on its high value as waterbird habitat, specifically the presence of many migratory species and more than 1% of the population of four species (Lane et al, 2009; Australian Wetlands Database, 2010). The wetlands regularly support more than 30,000 waterbirds annually and 78 species have been recorded there.

Two major mosquito breeding areas have been identified in the Vasse-Wonnerup Ramsar wetland area (Wright, 1998):

- an area of samphire wetland near Ford Road, approximately 40 hectares.
- samphire wetland in the vicinity of Port Geographe, approximately 100 hectares.

Sampled wetlands

Six small wetlands located within the Vasse-Wonnerup Wetland System are included in the monitoring program (Figure 1), all adjacent to the main open water of the Vasse Estuary but with limited connectivity, and all in close proximity to the Port Geographe mosquito breeding area. Of these, three were included in the larvicide treatment area (treatment sites) and three were left untreated throughout the program (control sites). Suitable sites were limited by accessibility and a need for adequate depth to ensure sampling would be possible throughout the mosquito control period. Allocation as treatment and control wetlands was done with consideration of prevailing winds (predominantly south-westerly) to avoid drift of larvicide from treated areas.

The wetlands vary in size and shape, however are similar in terms of vegetation charactristics, are adjacent to the large open water area of the wetland system and not directly connected to each other or

the open water. Emergent vegetation at the edges of the wetlands consists of samphire (*Halosarcia*) and varying degrees of grassy weed species, mainly couch (*Cynodon*). Submerged vegetation is dense within the wetlands and includes submerged *Halosarcia*, *Lamprothamnium papulosum* and *Ruppia megacarpa*. Filamentous green algae (Cladophora) is also present at several sites, at times growing very densely (sites T2 and C2). While generally similar, variation in characteristics of depth, water quality, extent of emergent and submerged aquatic plant growth, and seasonal algal growth have consequences for the macroinvertebrate community in each wetland. Replication of sampling across different wetlands is important in assessing impacts of larvicide treatment in the context of natural variation to ensure we are not simply comparing two wetlands which naturally support different macroinvertebrate communities. In addition, replication buffers against unforeseen problems or changes that may occur.

The sampled wetlands are shallow, with a maximum depth of 628mm (T3), and water depth during sampling is dependent on seasonal rainfall (Table 2). Sites C2 and C3 were the shallowest and site C3 was dry during one application in December 2010. Average temperature values were similar across sites (Table 2), and temperature measurements varied with the time of sampling during the day and weather conditions. All sites were brackish, but conductivity varied across sites with C3 and T3 having the highest values (Table 2). pH values were generally high (Table 2), likely due high levels of photosynthesis by plants and algae in the wetlands during the day, which removes carbon dioxide from the water. Dissolved oxygen values were consistently very high, further indicating high rates of photosynthesis within the water column.

Table 2. Mean depth range and mean values for physical parameters for each sampled wetland.

Site	Wetland depth range (mm)	Temperature (^{O}C)	Conductivity (mS/cm)	pH	Dissolved oxygen (mg/L)
T1	430-628	22.6	8.73	9.35	14.3
T2	364-562	22.9	9.57	9.62	17.3
T3	265-463	22.8	17.01	9.34	19.1
C1	360-558	22.5	11.70	9.31	15.4
C2	82-280	23.4	10.33	9.05	21.6
C3	72-270	23.9	20.70	9.41	19.5

2.2 Indicator organisms

Aquatic invertebrates of Order Amphipoda and Class Ostracoda are listed as indicator organisms in the conditions of approval for larvicide use. These groups were again the most common macroinvertebrate groups found in samples, and so are valuable indicator groups. In assessing ecological impacts on wetlands it is useful and interesting to consider the whole aquatic invertebrate community structure, and so all organisms collected in sampling were identified and counted.

2.3 Non-target invertebrate sampling

Aquatic invertebrate samples were collected from each study wetland within forty-eight hours before; and between twenty-four and forty-eight hours after larvicide application. On each sampling occasion, one sweep sample was taken at each treatment and control site. Sweep samples were collected from 10-metre transects using a long-handled D-frame sweep net with 250µm mesh size and 370mm width. The net was moved up and down along transects through the water column above the sediments and through submerged vegetation. This is a common method for wetland invertebrate sampling (Chessman et al, 2002; Davis et al, 1997). Sample collection points were selected randomly along the accessible shoreline, and 10-metre transects measured through areas deep enough for use of the sweep net, and within or as close to emergent vegetation as possible. As emergent samphire marshes are the primary problem mosquito areas and targeted by larvicide applications, this habitat was included within transects as much as practical.

Sweep net samples were washed thoroughly with clean water on site and vegetative material removed as much as practical. All samples were preserved in ethanol on site and stored below 4°C prior to processing in the laboratory. In the laboratory, samples were further cleaned prior to identification and counting of invertebrates. Processing included sorting and counting of organisms, using a dissecting microscope and counting trays as required for small macroinvertebrates and zooplankton. Volume-based subsampling was undertaken for groups present in very large numbers.

One sample was taken from each of three control and three treatment wetlands on each sampling occasion. This approach was preferred to taking three replicates from within only one treatment and one control wetland due to potential bias associated with particular characteristics of those wetlands, and the risk of having no data available if unplanned problems occurred with one site. While replicate samples from within each wetland would be ideal, this is difficult to achieve due to time constraints and short notice for sampling; and budget limitations associated with time-consuming sample processing[1].

[1] Replicate samples within two treatment and two control sites were included in 2012 sampling. This highlighted the potential for variation both within treatment and control groups and within each wetland. Average values for each site were used for the present analysis.

2.4 Efficacy sampling

Since the commencement of mosquito control operations in the Vasse Wonnerup Wetlands, Environmental Health Officers from the City of Busselton have undertaken post treatment sampling of mosquito larvae to assess the efficacy of larval treatments. For treatments with S-Methoprene, multiple mosquito larvae are collected from various treatment sites within the wetlands and kept in beakers over a period of 10 days to observe emergence rates. For *Bti* treatments, larval dipping is undertaken within the wetlands 1 – 2 days before and after each treatment, to allow comparison of pre- and post- treatment larval densities.

2.5 Data analysis

Statistical comparison of treatment and control sites in related to aerial larvicide application was done using repeated-measures analysis of variance (ANOVA), which is appropriate because the same sites were sampled multiple times and are therefore repeated measures. Because control sites were not treated, control sample data was aggregated for each treatment date. Thus there were three sample types for the ANOVA: pre-treatment, post-treatment and control. Sample type was the between-subjects factor (3 levels: pre-treatment, post-treatment and control); and the repeated-measures factor was larvicide application date (19 levels for S-methoprene, and 3 levels for *Bti*, corresponding to each application). ANOVA was completed for abundance of amphipods (1 species – *Austrochiltona subtenuis*), ostracods (*Mytilocypris* spp. and others as two groups), zooplankton (Copepoda and Cladocera), combined abundance of other macroinvertebrate groups, and taxa richness (family-level or above). Pairwise comparisons provided further information on differences between pairs of sample types and treatments.

Valid use of ANOVA assumes that data is normally distributed, that variance within groups is similar (homogeneous) and, that variance is similar between levels of repeated measures. Kolmogorov-Smirnov and Shapiro Wilk tests indicated richness data was normally distributed, but abundance data for all taxa required transformation ($\log_{10} + 1$) to meet normality assumptions for ANOVA (this reduces the effect of outlier and extremes in data). Levene's test verified homogeneity of variance, and Mauchly's test confirmed equal variances between different levels of measurement (sphericity). These analyses were performed using SPSS statistical software.

Differences in aquatic invertebrate community assemblage between sample types were further analysed by multivariate procedures using abundance data for all taxa. Two-way crossed ANOSIM (analysis of similarity) tested how closely each of the three sample types resembled the others, using the factors of sample type and application number. The SIMPER procedure (similarity percentages, 2-way crossed design) was performed to identify the families responsible for the differences between groups identified in ANOSIM. Abundance data was transformed for these analyses ($\log_{10} + 1$). All

multivariate analyses were completed using software package PRIMER 6 (Plymouth Routines in Multivariate Ecological Research: Clarke and Warwick, 2001).

Interpreting statistical tests

Statistical procedures test hypothesis about the data, generally the *null hypothesis*: that there is no difference between groups of data. The significance level for testing is commonly set at 0.05, as used in this study. Statistical procedures produce a test statistic: the F value in ANOVA and the R value in ANOSM; and a *P* value for this statistic. The *P* value, which is "the probability of observing results as extreme (or more) as those observed if the null hypothesis is true" (Field 2005). This value is between 0 and 1: If *P* is very low, the null hypothesis is less likely to be true (i. e. there is a difference between groups), while a higher *P* indicates it is more likely that there is no difference. If *P* is less than the significance level (0.05), the null hypothesis is rejected and the difference between groups is considered significant. We can have confidence in this decision, because there is only a very small chance (5%) that we would have obtained these results if there was no difference.

3 Results

A total of 30 taxa were identified at family level or above, including over 43 species (Table 3) over the sampling period September 2009 to December 2015. Although some taxa were identified to genus or species level, they were counted mostly at the level of family or above. Ostracoda were identified to species level and counted as two groups: *Mytilocypris* spp. and other Cyprididae ostracods. Taxa present in samples varied across years, but the most abundant groups that were present consistently in all years included Amphipoda, Ostracoda, Copeoda, Cladocera, Dytiscidae, Hydrophilidae, Corixidae, Notonectidae, Chironomidae, Culicidae and Pomatiopsidae. Mean abundances of invertebrate taxa were of a similar magnitude for treatment and control wetlands, as was taxa richness and species composition. The following sections describe detailed results for abundance of each of the indicator groups, Amphipoda and Ostracoda, as well as for Chironomidae, Zooplankton (Copepoda and Cladocera), and other macroinvertebrate taxa; for community composition; and for efficacy of larvicide use.

Table 3. Taxa encountered during aquatic invertebrate sampling 2009 – 2015 and mean abundance (individuals per sample) in treatment and control wetlands.

Classification			Mean abundance Treatment sites	Mean abundance Control sites
Order Amphipoda	Family Ceinidae	*Austrochiltonia subtenuis*	3143 (740)	2465 (453)
Class Ostracoda	Family Cyprididae	*Mytolocypris* spp.	508 (85)	1060 (115)
		M. ambiguosa		
		M. tasmanica chapmani		
		Other	1704 (397)	3160 (440)
		Alboa worooa		
		Cypridopsis funebris		
		(3 other species)		
Class Copepoda	Order Cyclopoida		12711 (3113)	15739 (1930)
	Order Calanoida			
Suborder Cladocera			16620 (2708)	18541 (2146)
Order Collembola			0.4 (0.1)	0.3 (0.1)
Order Decapoda	Family Palaemonidae	*Palaemon australis* (shrimp)	12 (5)	7 (3)
Class Insecta	Order Coleoptera	Curculionidae	5 (3)	3 (2)
	(Beetles)	Dyticidae	24 (5)	24 (3)
		Cybister sp.		
		Megaporus Sp.		
		Necterosoma sp.		
		Rhantus sp.		
		Hydrophilidae	27 (4)	24 (3)
		Berosus sp.		
		Haliplidae	0.4 (0.1)	0.4 (0.1)
		Scirtidae	<0.1	<0.1
	Order Hemiptera	Corixidae	24 (8)	9 (4)
	(Bugs)	*Agraptocorixia* sp.		
		Micronecta robusta		
		Notonectidae	58 (15)	58 (11)
		Anisops sp.		
	Order Trichoptera	Hydroptilidae	0.1 (0.1)	2 (0.6)
	(Larval caddisflies)	Leptoceridae	1 (1)	2 (1)
	Order Diptera	Chironomidae	642 (204)	393 (116)
		Chironomus sp.		
	(Larval flies)	Stratiomyidae	10 (4)	2 (2)
		Culicidae	17 (6)	46 (10)
		Aedes sp.		
		Culex sp.		
		Ephydridae	5 (4)	24 (6)
		Other	10 (6)	31 (8)
Order Odonata	Suborder Zygoptera	Coenagrionidae	1 (0.2)	1 (0.2)
	(Larval damselflies)	Lestidae	1 (0.4)	1 (0.3)
	Suborder	Aeshnidae	0.5 (0.2)	0.5 (0.1)
	Epiproctophora	Corduliidae	0	<0.1
	(Larval dragonflies)	Libellulidae	0	<0.1
Class Gastropoda		Pomatiopsidae	72 (23)	101 (24)
		Coxiella striata		
		Planorbidae	<0.1	<0.1
		Hydrobiidae	<0.1	<0.1
		Potamopyrgus sp.		
		Lymnaeidae	<0.1	<0.1
Class Clitellata	Hirudinea		16 (14)	9 (8)
Class Arachnida	Order Acarina	Hydracarina (water mites)	1 (0.6)	1 (0.3)

3.1 Ostracoda

Ostracods were found at high abundance throughout the study period, accounting for 47% of total macroinvertebrate abundance, occurring in 95% of samples with an overall average of 3203 individuals per sample. This high abundance suggests ostracods are a potentially important food source for waterbirds and therefore valuable indicators for this monitoring program. Abundance varied greatly both within and between sites, and over time (shown by large variation in mean and wide error bars in Figures 4, 5, and 6). Seven species of Ostracods were encountered during sampling, all belonging to the family Cyprididae: *Mytilocypris tasmanica chapmani*, *Mytilocypris ambiguosa*, *Alboa worooa* (Figure 2), and Cyprididae sp. 2 were the most common species and were found in all sample years. *Cypridopsis funebris* occurred only in two samples during 2010, and Cyprididae sp. 3, Cyprididae sp. 4 were found only during 2010 and 2012 sampling. While relative abundance of ostracod species varied between years, overall *Mytilocypris* spp. accounted for 24% of total ostracod abundance for the 7-year period, Alboa and Cyprididae 2 for 74%, and other species for only 2%. Ostracod data were analysed as two groups: *Mytilocypris* spp. and other Cyprididae ostracods.

Figure 2. Common ostracods found during sampling throughout 2009 to 2015: Mytilocypris tasmanica chapmani (a), Mytilocypris ambiguosa (b), and Alboa worooa (c).

3.1.1 Response to S-methoprene

Simple face-value comparison of data for pre- and post- application samples from treatment sites and control site samples did not indicate any consistent patterns in *Mytilocypris* spp. abundance with respect to larvicide application (Figure 3). Within treatment sites, lower abundance following larvicide application occurred in only six of nineteen treatments. Although mean abundance was higher in control sites for several applications from 2009 – 2011, this was not the case for following years of sampling. Overall mean abundance was highest in control samples (1061 individuals per sample), lowest in pre-treatment samples (518), and slightly higher in post-treatment samples (642), however ANOVA found no significant effect of sample type on *Mytilocypris* spp. abundance ($F_{2,6} = 0.09$, $P = 0.915$) and no difference between any combination of site types (pairwise comparisons $P > 0.722$). These *P-values* close to and equal to 1 provide very high confidence in this outcome of no

effect. A highly significant effect of application number was shown ($F_{18,36}$ = 4.1, P < 0.01), and this was the case for all three sample types, with no interaction effect between application and sample types ($F_{36,108}$ = 0.8, P = 0.784). This dependence on the date of application suggests that variation in abundance over time was important. These results do not provide any evidence of negative effects of S-methoprene on *Mytilocypris* spp. abundance.

Abundance of other Cyprididae species was also unaffected by S-methoprene application (Figure 4). Although control site samples had a higher overall mean abundance compared to treatment sites (3668 individuals per sample), mean values for pre- and post -treatment samples were similar (1789 and 2013, respectively). There was no significant different in abundance between sample types ($F_{2,6}$ = 0.43, P = 0.671) and all pairwise comparisons between sample types were also insignificant (P > 0.402). As for *Mytilocypris* spp., the effect of application number was highly significant ($F_{18,36}$ = 4.1, P < 0.01), regardless of sample type (no interaction effect of application and sample types: $F_{36,108}$ = 0.67, P = 0.913). Abundance of other Cyprididae species appears to be dependent on variation over time for all sample types, with no effect of S-methoprene indicated.

3.1.2 Response to *Bti*

Ostracod abundance data did not indicate any consistent or significant response to application of *Bti* (Figure 5). *Mytilocypris* spp. abundance was lower in post-treatment samples compared to pre-treatment samples following applications in September 2014 and October 2015, but increased following the application in December 2014 (Figure 5b). There was no significant effect of sample type ($F_{2,6}$ = 0.17, P = 0.847), and pairwise comparisons were also non-significant (P > 0.589). Abundance differed significantly between applications ($F_{2,12}$ = 4.6, P = 0.033) for all sample types (no interaction effect: $F_{4,12}$ = 1.0, P = 0.444).

Cyprididae abundance did not differ significantly between sample types during *Bti* applications ($F_{2,6}$ = 0.65, P = 0.557, pairwise comparisons P > 0.303). There was a significant effect of application ($F_{2,12}$ = 10.32, P = 0.002), owing to lower overall abundance during the second application, but there was no interaction effect ($F_{4,12}$ = 1.30, P = 0.206) because all sample types changed in a similar way over time (i. e. decrease followed by increase, Figure 5b).

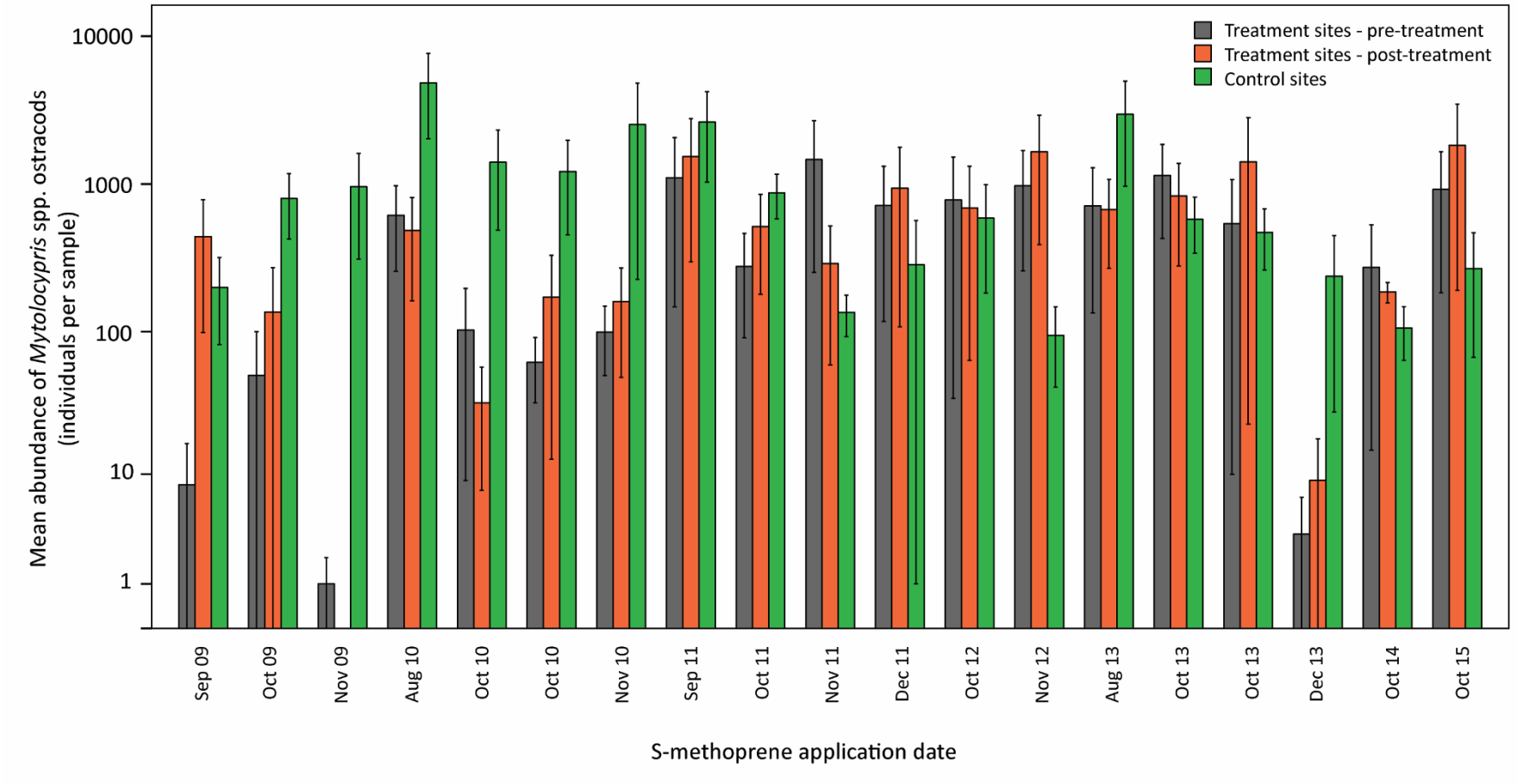

Figure 3. Mean abundance of ostracods *Mytilocypris tasmanica chapmani* and *Mytilocypris ambiguosa* in pre- and post-treatment samples from treatment sites and in control site samples for each application of S-methoprene in the Vasse-Wonnerup Wetland 2009-2015. Error bars are standard error.

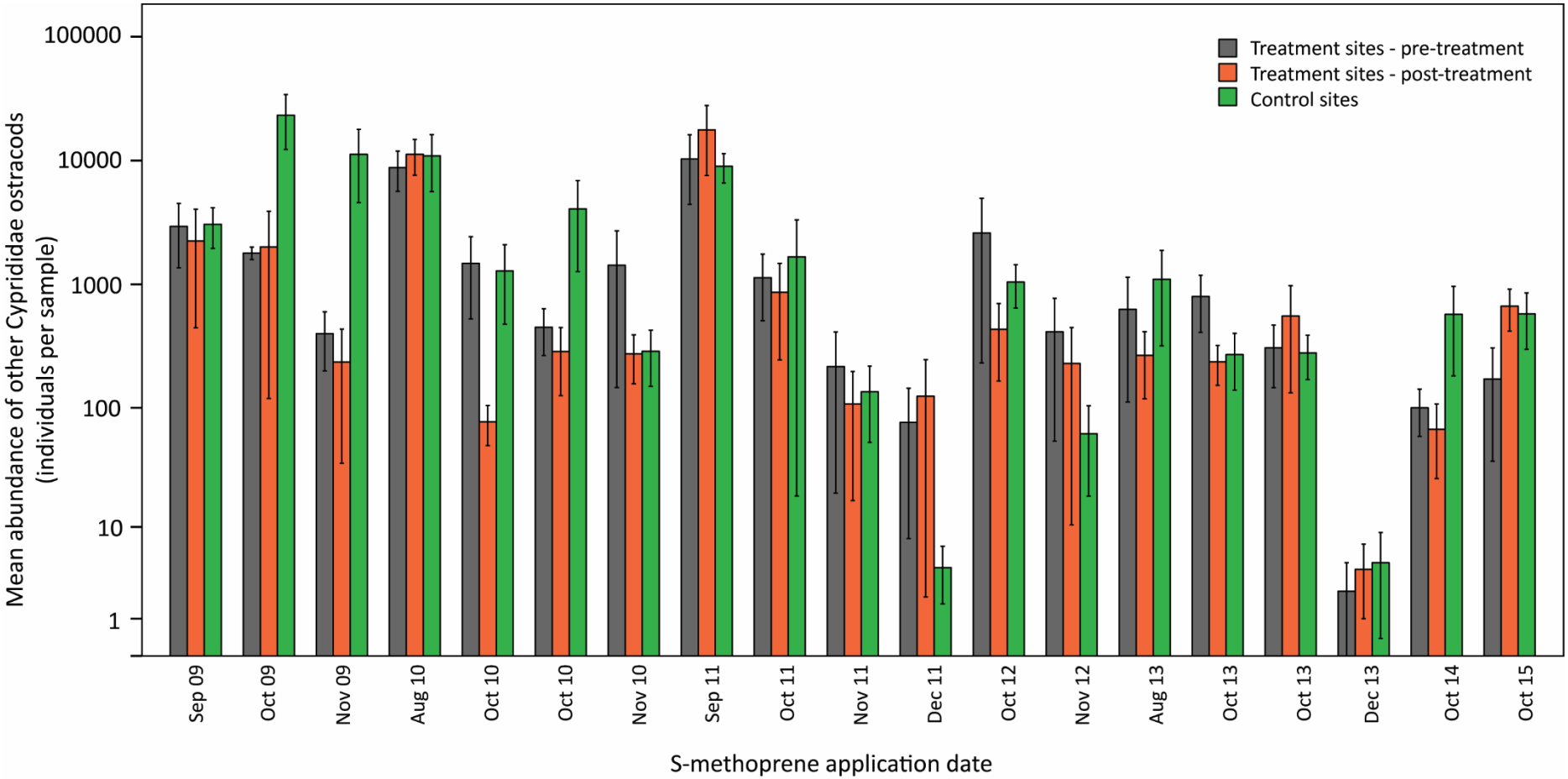

Figure 4. Mean abundance of ostracods excluding *Mytolocypris* spp. in pre- and post-treatment samples from treatment sites and in control site samples for each application of S-methoprene in the Vasse-Wonnerup Wetland 2009-2015. Error bars represent standard error.

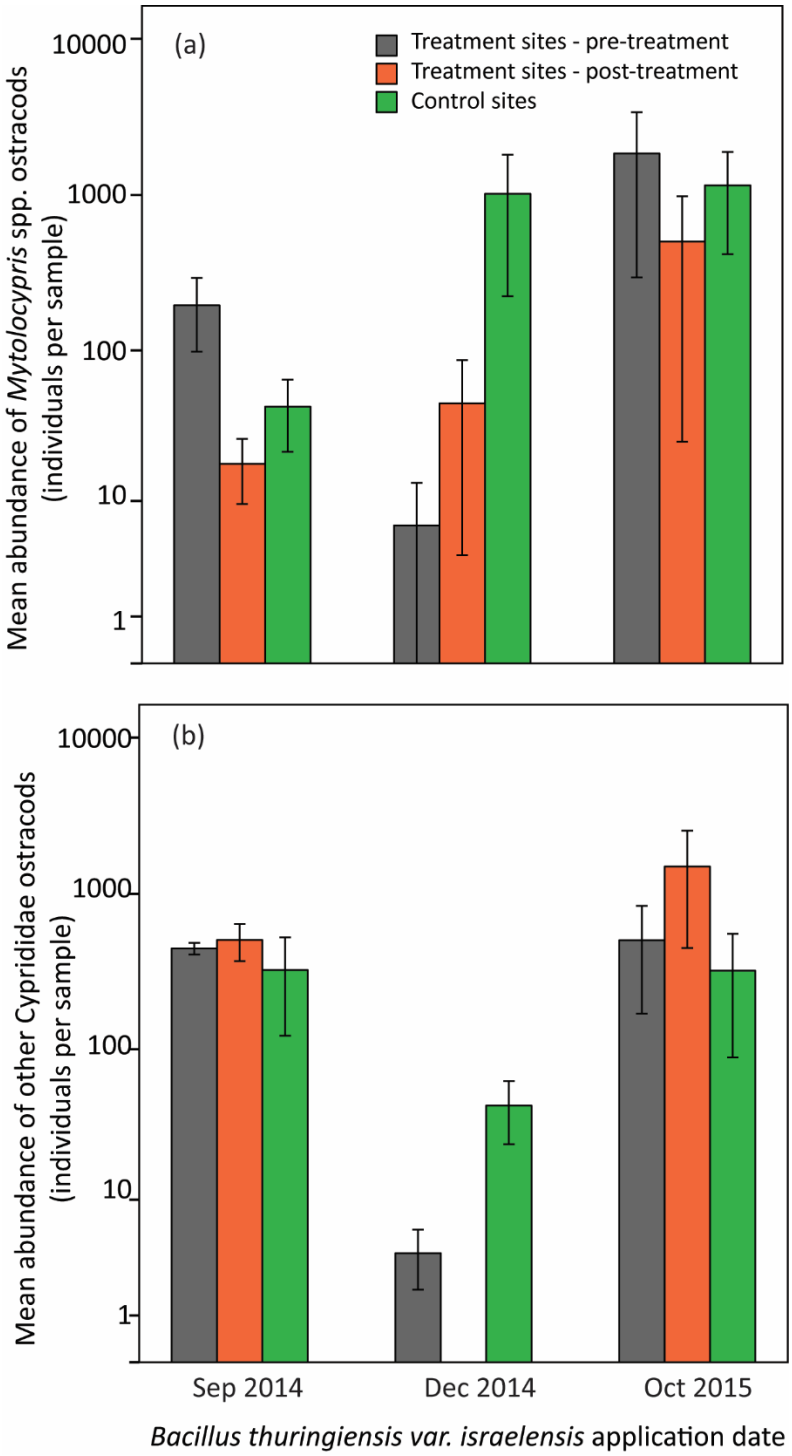

Figure 5. Mean abundance of Mytilocypris spp. (a) and other Cyprididae (b) ostracods in pre- and post-treatment samples from treatment sites and in control site samples for each application of *Bacillus thuringiensis var. israelensis* in the Vasse-Wonnerup Wetlands 2009-2015. Error bars represent standard error.

3.2 Amphipoda

One species of amphipod was encountered during sampling from 2009 - 2015: *Austrochiltonia subtenuis* from the family Ceinidae (Figure 6). This species was consistently present throughout all years, being present in 92% of samples. Amphipod abundance was high, with an overall mean of 2803 individuals per sample, although varied greatly both within and between sites, and over time (shown by large variation in mean and wide error bars in Figures 7). Variation of amphipods was greater than ostracods. Samples containing very high abundance of amphipods consisted of mainly small individuals, while samples containing mostly large individuals has lower abundance. *Austrochiltonia subtenuis* accounted for 41% of all macroinvertebrates collected in sampling, and so this species and ostracods were the dominant groups found in samples. Its high abundance and common present in all sites makes it important for inclusion as an indicator species that is a likely food source for waterbirds.

Figure 6. The amphipod *Austrochiltonia subtenuis.*

3.2.1 Response to S-methoprene

The variation in abundance of *A. subtenuis* between site types did not reflect any response to S-methoprene larvicide applications (Figure 7) and there was no significant difference in abundance of the three sample types: pre-treatment, post-treatment and control samples ($F_{2,6} = 0.05$, $P = 0.95$). As for ostracods, abundance of this species showed a highly significant effect application date ($F_{18,108} = 9.05$, $P < 0.01$). Within each sample date, abundance was often similar across sample types, and mean values appeared to fluctuate similarly over time (Figure 7), with no significant application x sample type interaction effect.

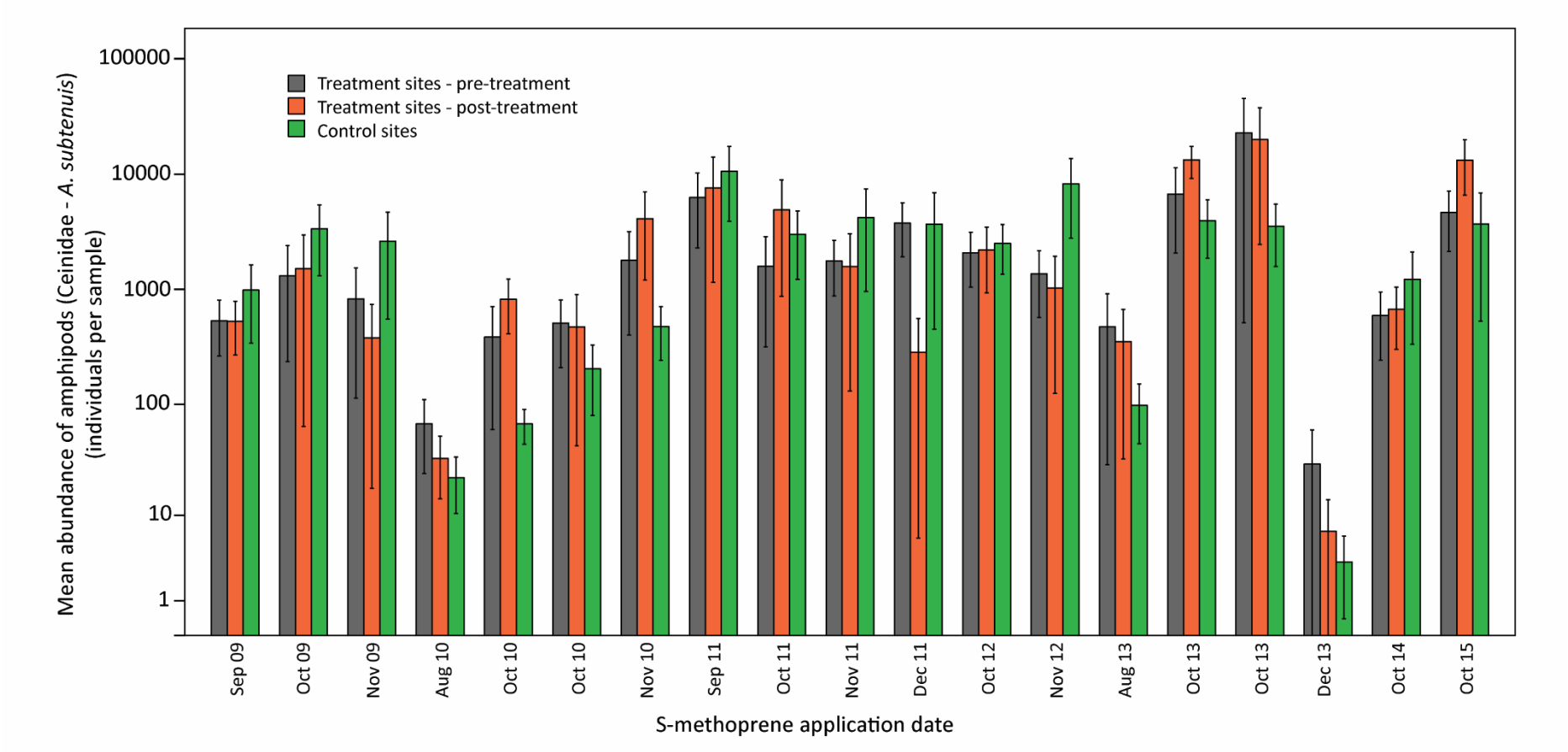

Figure 7. Mean abundance of amphipod Austrochiltonia subtenuis in pre- and post-treatment samples from treatment sites and in control site samples for each application of S-methoprene in the Vasse-Wonnerup Wetlands 2009-2015. Error bars represent standard error.

3.2.2 Response to *Bti*.

Amphipod abundance in samples taken during *Bti* applications varied greatly within pre- and post-treatment samples and control samples (Figure 8) and the three sample types did not differ significantly ($F_{2,6} = 0.21$, $P = 0.816$; pairwise comparisons $P > 0.556$). While mean abundance was lower after *Bti* application in December 2014 and October 2015, but this was not consistent for all sites and post-treatment abundance was higher following the first treatment in September 2014. Overall mean amphipod abundance was higher in post-treatment samples (3903) compared with pre-treatment (3022) and control (2798) samples. There was no significant difference in abundance between application dates ($F_{2,12} = 2.56$, $P = 0.118$).

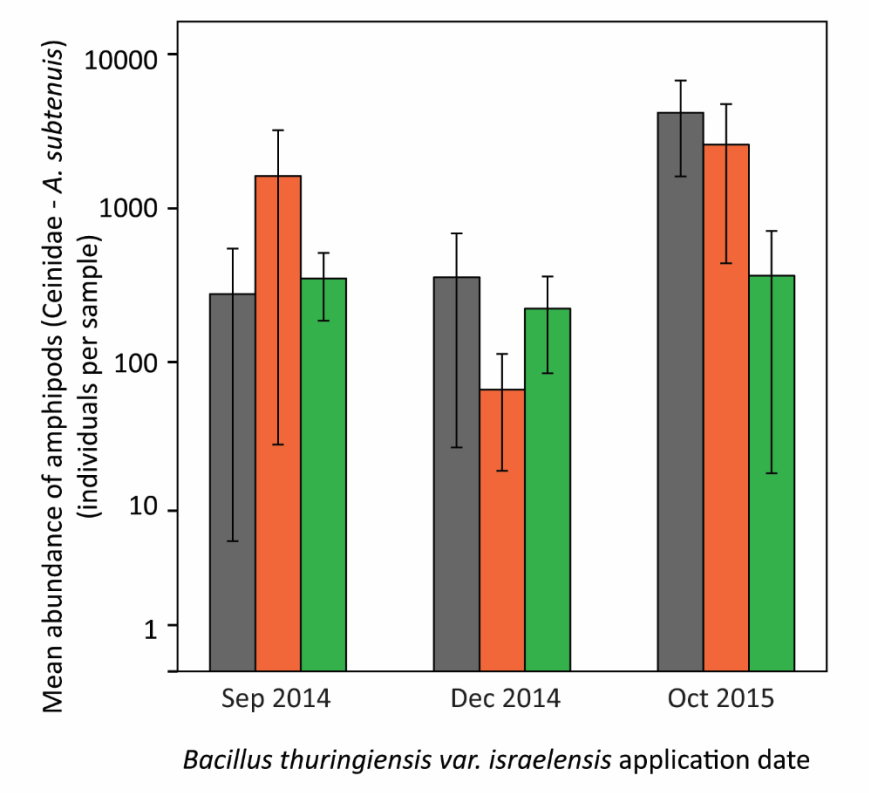

Figure 8. Mean abundance of amphipods in pre- and post-treatment samples from treatment sites and in control site samples for each application of *Bacillus thuringiensis var. israelensis* in the Vasse-Wonnerup Wetlands 2009-2015. Error bars represent standard error.

3.3 Chironomidae

Excluding indicator taxa, the most prevalent macroinvertebrate family encountered was Chironomidae (non-biting midge larvae, Figure 9), predominantly *Chironomus* sp., with an overall mean abundance of 517 individuals per sample. Although only accounting for 8% of macroinvertebrate abundance, Chironomids were common at all sites and present in 75% of all samples, with many samples having abundance greater than 1000. Chironomidae abundance was extremely variable over the study period (Figure 10). Although not specified as an indicator group, Chironomids are of interest in this study because of their relatively high abundance and because they are in the same Order (Diptera) as mosquitos (Culicidae). In particular, *Bti* specifically targets Diptera larvae.

Figure 9. *Chironomus* sp. larvae were the dominant Chironomidae found in samples. (Image source: Steve Hopkin / www.ardea.com).

3.3.1 Response to S-methoprene

It is clear from a visual comparison of mean values for pre- and post-treatment and control samples that abundance of chironomids in samples was not related to S-methoprene larvicide application (Figure 10). Post-treatment abundance was variably high and lower than pre-treatment abundance, and abundance was lower in control sites than treatment sites for most application dates. As would be expected giving these results, sample type had no significant effect on chironomid abundance ($F_{2,6}$ = 0.12, P = 0.888; pairwise comparisons P > 0.675). Abundance clearly varied greatly among larvicide application dates, and this was highly significant ($F_{18,36}$ = 7.98, P < 0.001). All sample types were similarly affected by application date (no interaction effect: $F_{36,108}$ = 1.32, P = 0.140), and overall means were similar (pre-treatment: 587, post-treatment: 441, control: 460).

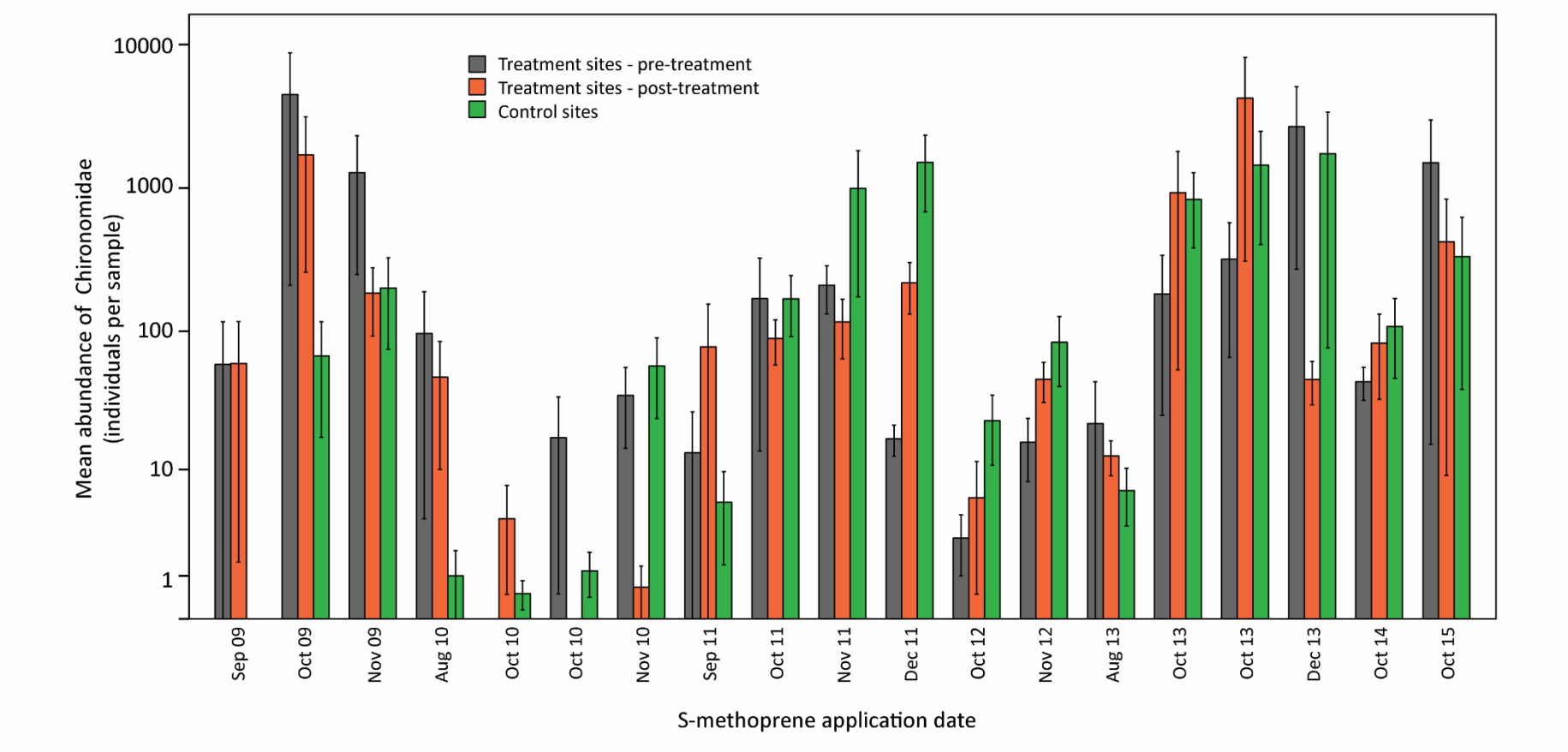

Figure 10. Mean abundance of Chironomidae in pre- and post-treatment samples from treatment sites and in control site samples for each application of S-methoprene in the Vasse-Wonnerup Wetlands 2009-2015. Error bars represent standard error.

3.3.2 Response to *Bti*

Chironomid abundance was consistently (though not significantly) higher in post-treatment samples compared to pre-treatment samples for all applications of *Bti* (Figure 11). Abundance was much higher in control site samples compared to both pre-and post-treatment samples during *Bti* application in September 2014, but was similar or lower for other application dates. Overall mean abundance in post-treatment samples was much higher (2266) than for other types (pre-treatment: 608, control: 366), but there was no significant different in abundance between the three sample types ($F_{2,6} = 0.03$, $P = 0.974$; note we can have high confidence in this outcome because the P-value is close to 1). Application date did have a significant effect on abundance for all sample types ($F_{2,12} = 18.05$, $P < 0.001$) and pairwise comparisons indicated this resulted from lower abundance during the September 2014 application ($P < 0.004$) (Figure 11), while abundance did not differ significantly between the other two application dates ($P = 0.091$).

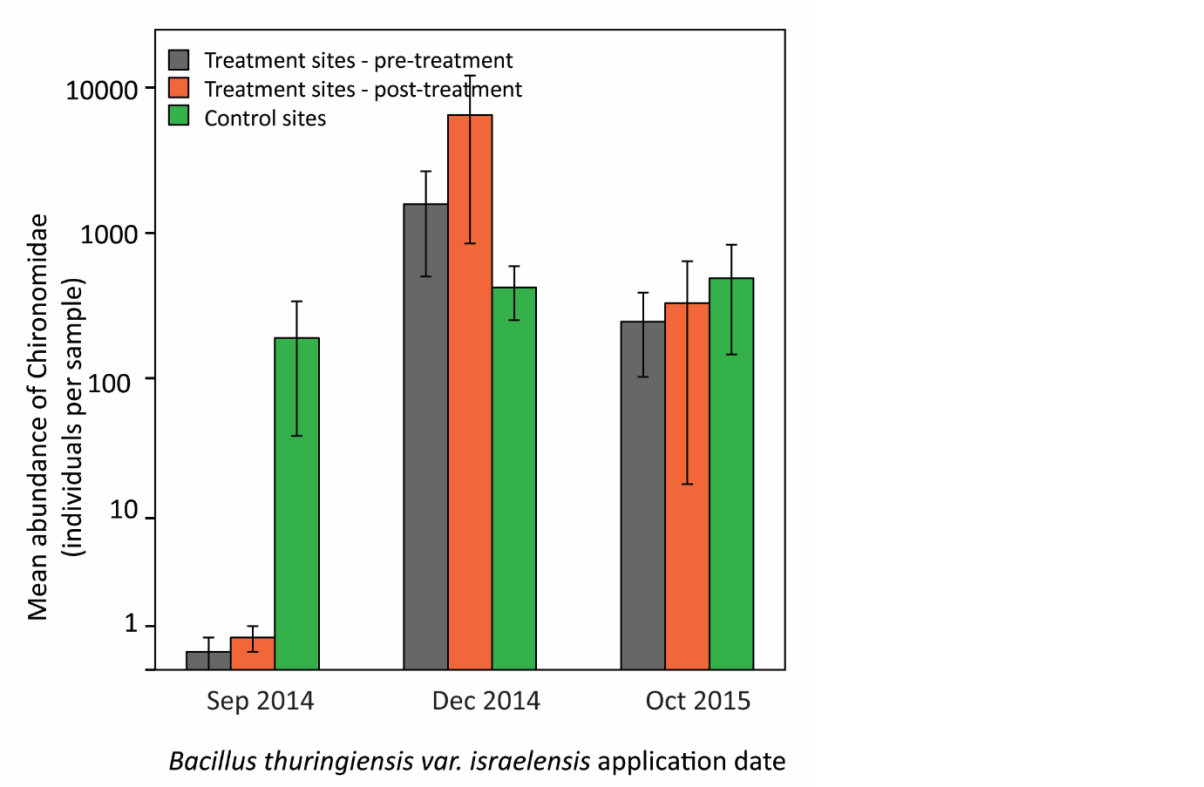

Figure 11. Mean abundance of Chironomidae in pre- and post-treatment samples from treatment sites and in control site samples for each application of *Bacillus thuringiensis var. israelensis* in the Vasse-Wonnerup Wetlands 2009-2015. Error bars represent standard error.

3.4 Other macroinvertebrate groups

Excluding Amphipoda, Ostracoda and Chironomidae, 27 other macroinvertebrate families were found in samples from 2009 to 2015, but these contributed to only 5% of overall abundance (mean = 317 individuals per sample). The most common families were (in order) Pomatiopsidae (*Coxiella striata*), Notonectidae (*Anispos* sp.), Culicidae, Hydrophilidae, Dytiscidae and Corixidae (Figure 12, Table 3). Both adult and larvae Coleoptera were observed, but abundance of larvae was higher than adults for both Dytiscidae (98% larvae) and Hydrophilidae (79% larvae).

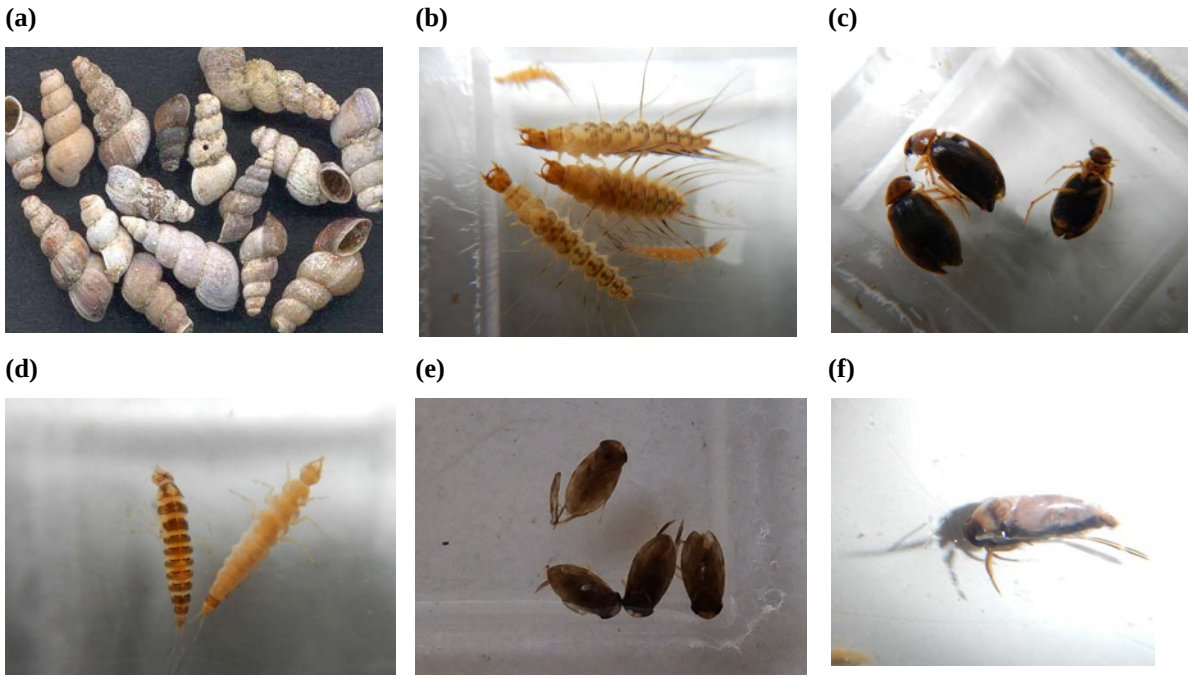

Figure 12. Some of the most common macroinvertebrates found during 2013 sampling: Pomatiopsidae gastropods (*Coxiella sp.*) (a), Hydrophylidae beetle larvae (b) and adults (c), Dyticidae beetle larvae (d), Corixidae (water boatmen, e) and Notonectidae (backswimmers, f).

Image sources for (a) http://www.jandmgrist.com/images/fullsize/Coxiella_striata.jpg

3.4.1 Response to S-methoprene

Total abundance of these other macroinvertebrate groups was less variable during S-methoprene applications than found for ostracods, amphipods and chironomids, both over time and between sample types (Figure 13). Overall means were similar for pre-treatment samples (300), post-treatment samples (291) and control samples (334). No consistent decrease in abundance between pre- and post-treatment samples or between treatment and control samples was observed, and there was no significant difference between sample types ($F_{2,6} = 0.12$, $P = 0.888$; pairwise tests $P > 0.649$). Application date did have a significant effect on abundance ($F_{18,36} = 2.36$, $P = 0.003$) and all sample types varied similarly over time (no application x sample type interaction: $F_{36,108} = 0.82$, $P = 0.753$). This results suggest S-methoprene application did not adversely affect total abundance of these macroinvertebrates.

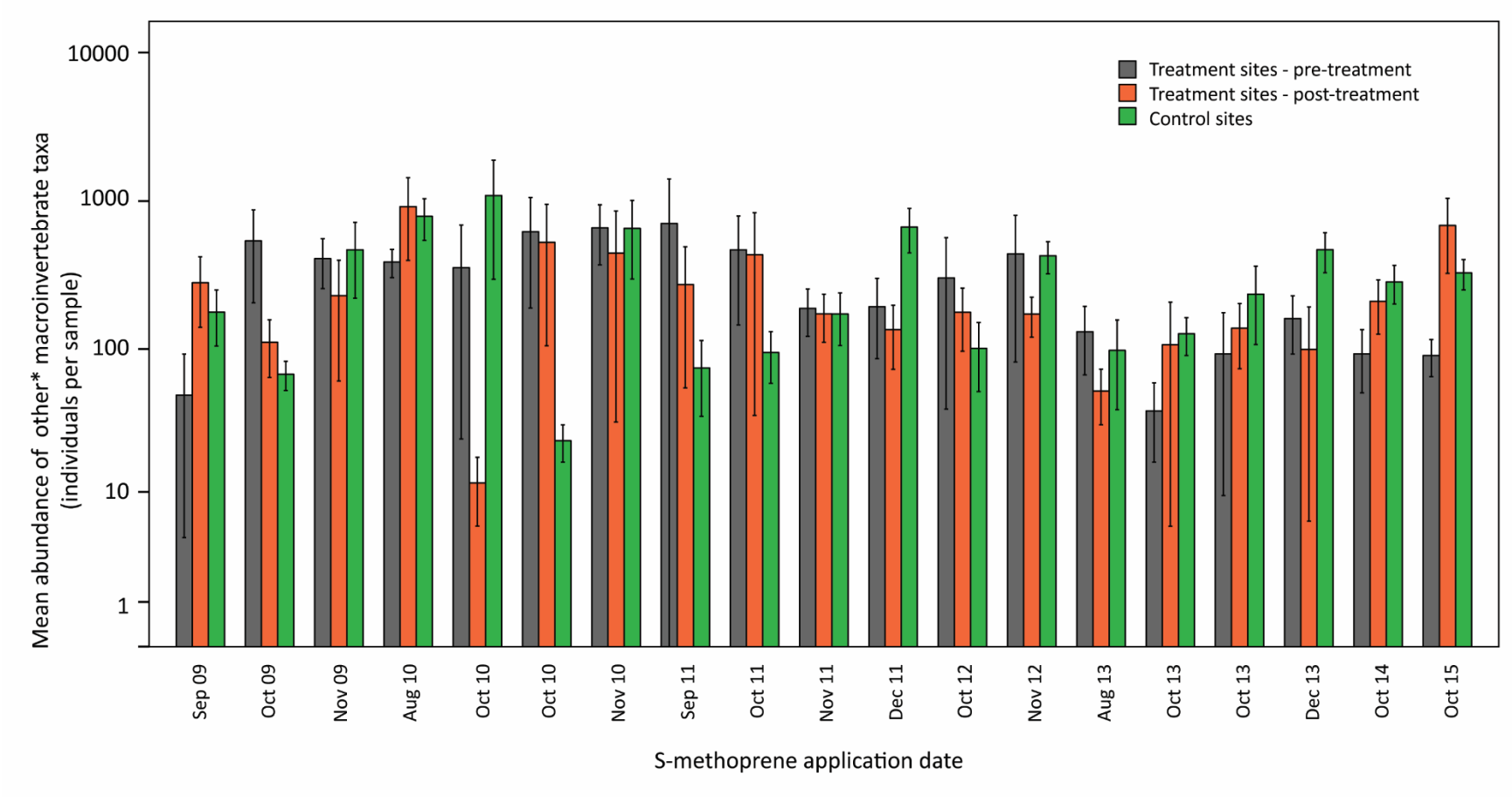

Figure 13. Mean abundance of other macroinvertebrate taxa in pre- and post-treatment samples from treatment sites and in control site samples for each application of S-methoprene in the Vasse-Wonnerup Wetlands 2009-2015. Error bars represent standard error. *This grouping excludes Ostracoda, Amhipoda, Chironomidae and zooplankton (Copepoda and Cladocera).

3.4.2 Response to *Bti*

During both applications of *Bti* in 2014, abundance of other macroinvertebrates was lower in post-treatment samples than in pre-treatment samples and control samples (Figure 14). In October 2015, post-treatment samples had the highest mean abundance. There was a significant effect of sample type on abundance of these macroinvertebrates ($F_{2,6} = 5.6$, $P = 0.04$), however pairwise comparisons showed significant differences between pre-treatment and control samples ($P = 0.020$) and post-treatment and control samples ($P = 0.034$) but not between pre- and post-treatment samples ($P = 0.701$). This suggests that treatment and control sites differed significantly, but not due to the *Bti* treatment. Lower mean abundance in post-treatment sites following the December 2014 application was owing to much lower Notonectidae abundance at two sites. There was also a significant effect of application date ($F_{2,12} = 4.9$, $P = 0.028$), owing to the difference in abundance between the two 2014 applications ($P = 0.013$), evident from the plotted data (Figure 14). Again, the data suggests that Notonectidae abundance was the source of this difference, with very high values for pre-treatment and control sites in December 2014. There was no strong evidence of a negative effect of *Bti* on abundance because there was no difference between pre-and post-treatment samples. The pattern of abundance differences seen between sample types for the December 2014 application would need to be consistent to provide strong evidence of an impact of *Bti*.

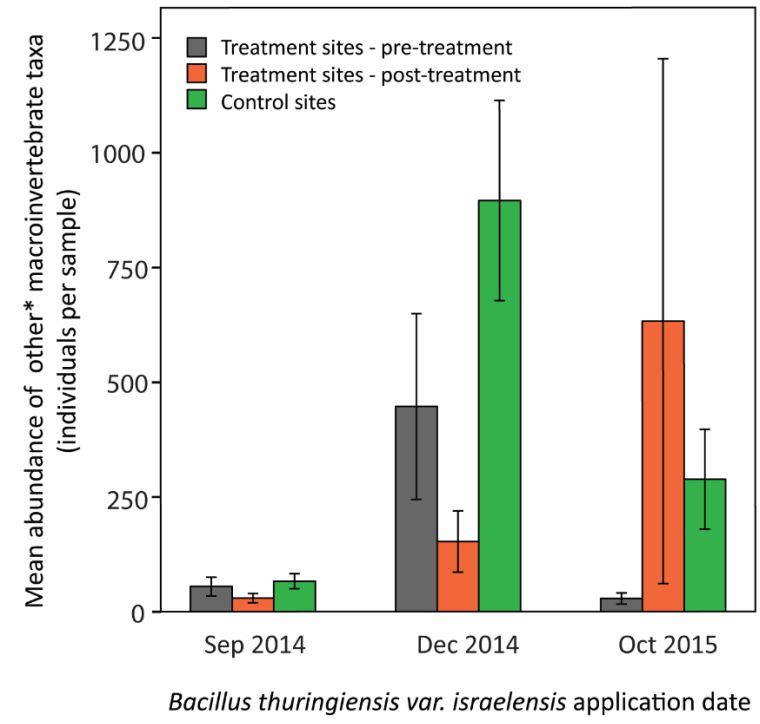

Figure 14. Mean abundance of other macroinvertebrate taxa in pre- and post-treatment samples from treatment sites and in control site samples for each application of *Bacillus thuringiensis var. israelensis* in the Vasse-Wonnerup Wetlands 2009-2015. Error bars represent standard error. *This grouping excludes Ostracoda, Amhipoda, Chironomidae and zooplankton (Copepoda and Cladocera).

3.5 Zooplankton

Zooplankton in this study includes microcrustaceans from Class Copepoda and Suborder Cladocera. These organisms were considered separately from macroinvertebrate groups in analysis of abundance data because they are often present in very large numbers and therefore may bias interpretation of results, and because this high abundance makes them interesting to analyse. They are likely to be an important component of the food web in these wetlands, providing a resource for waterbirds both directly, and indirectly as food for predatory invertebrates. Although total abundance of zooplankton was higher than indicator organisms, this equates to a substantially lower biomass owing to their small size. Both Calanoid and Cyclopoid copepods were observed, and *Daphnia* sp. were the dominant cladocerans (Figure 15). Different copepod groups were counted separately in 2009: 60% of these were Calanoida and 40% were Cyclopoida. Abundance of zooplankton over the 2009 – 2015 sampling period was 31 937 individuals per sample, with similar abundance in treatment and control sites (Table 3). Cladocera accounted for 55% of zooplankton and Copepoda accounted for 45%.

Figure 15. Common zooplankton in the Vasse-Wonnerup Wetlands: cyclopoid copepod (a), calanoid copepod (b) and the cladocerans *Daphnia magna* (c).

Image sources:
(a) http://university.uog.edu.172-31-22-36.previewmywsisite.com/botany/474/fw/copepods.htm
(b) http://www.photomacrography.net/forum/viewtopic.php?t=21235&sid=19e0317df50556994242e4d6eeb2ba4c
(c) https://upload.wikimedia.org/wikipedia/commons/c/c2/Daphnia_magna_asexual.jpg

3.5.1 Response to S-methoprene

Zooplankton abundance was slightly lower in treatment sites following the first four S-methoprene applications from September 2009 to October 2010, but both increases and decreases were observed during subsequent applications (Figure 16). Mean zooplankton abundance was similar for pre-treatment (33566), post-treatment (28240) and control (38930) samples and there was no significant difference between these sample types ($F_{2,6} = 0.41$, $P = 0.683$; pairwise comparisons $P > 0.426$). A highly significant effect of application ($F_{18,36} = 12.0$, $P < 0.001$) was found for all sample types (no interaction effect) as abundance was highly variable between application dates (Figure 16) . These results indicate that S-methoprene did not have a negative impact on zooplankton abundance.

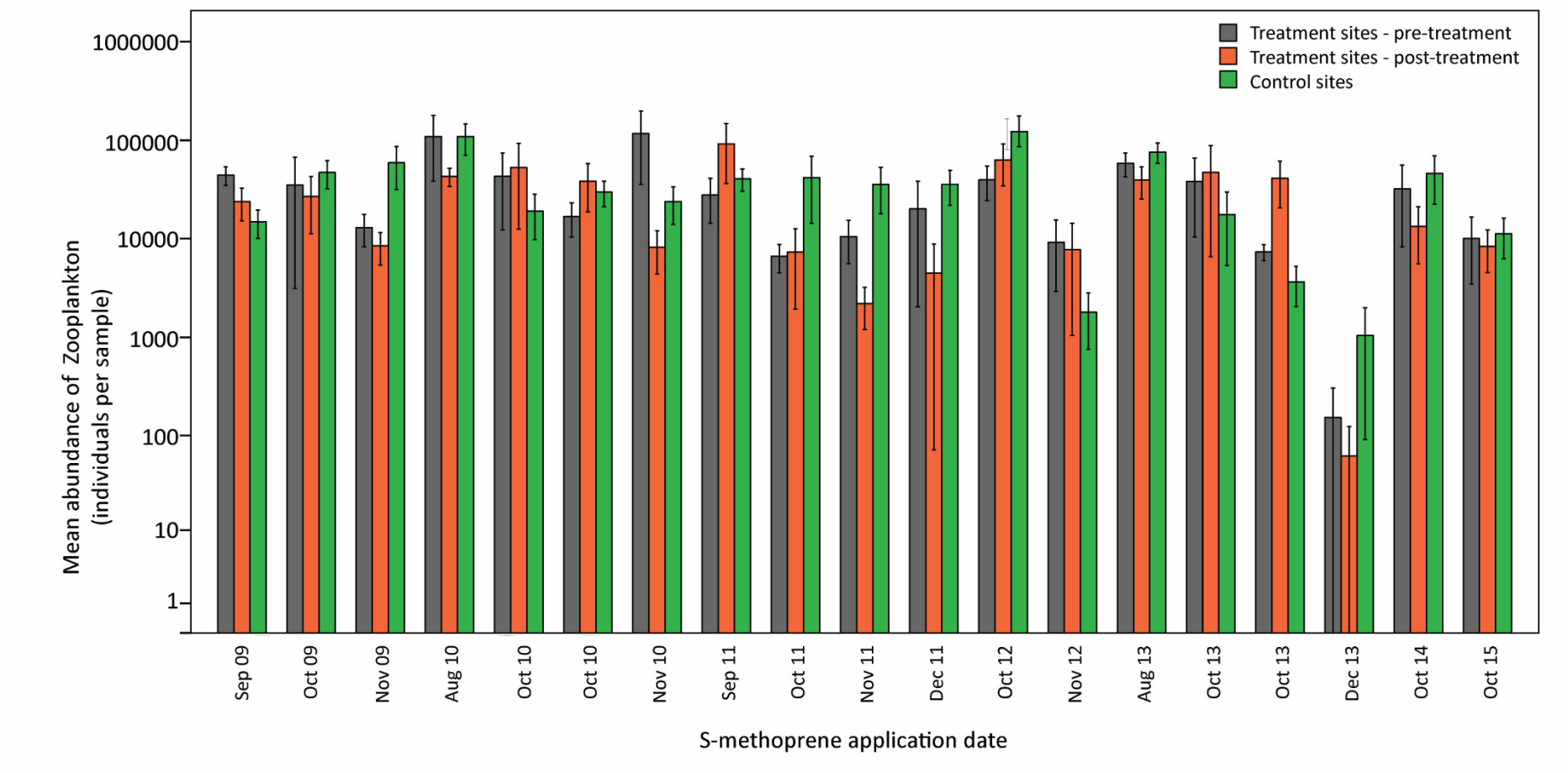

Figure 16. Mean abundance of zooplankton (Cladocera and Copepoda) in pre- and post-treatment samples from treatment sites and in control site samples for each application of S-methoprene in the Vasse-Wonnerup Wetlands 2009-2015. Error bars represent standard error.

3.5.2 Response to *Bti*

Comparison of pre-treatment, post-treatment and control sample data did not indicate any negative effect of *Bti* treatment, with mean abundances in treatment sites following each larvicide application higher or similar to other sample types (Figure 17). Overall mean abundance was substantially higher for post-treatment samples (35378) than pre-treatment (3253) or control samples (9153), however data were highly variable and there was no significant difference in abundance between the three sample types ($F_{2,6} = 0.87$, $P = 0.466$; pairwise comparisons $P > 0.243$). Abundance data did not vary greatly between applications of *Bti* for any sample type, with no effect of application date ($F_{2,4} = 3.13$, $P = 0.081$) or any application x sample type interaction ($F_{4,12} = 1.32$, $P = 0.140$).

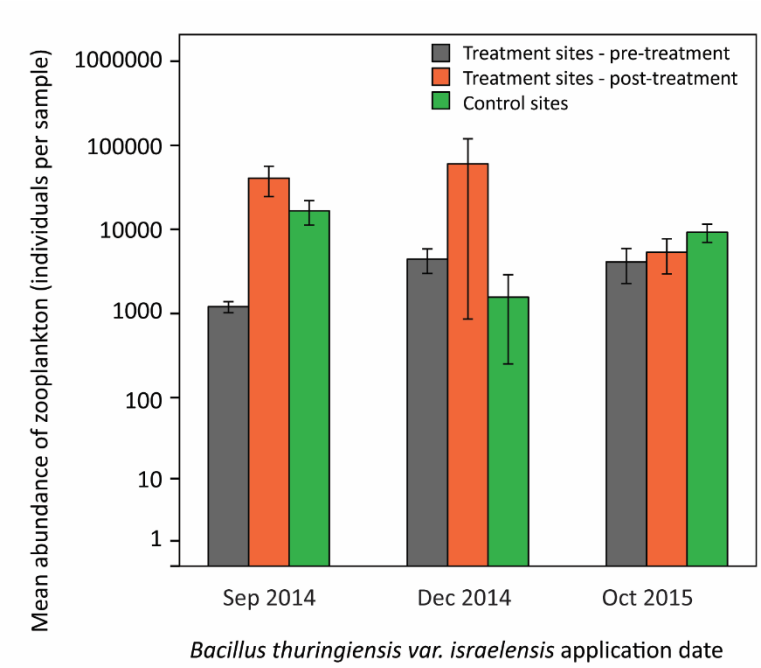

Figure 17. Mean abundance of zooplankton in pre- and post-treatment samples from treatment sites and in control site samples for each application of *Bacillus thuringiensis var. israelensis* in the Vasse-Wonnerup Wetlands 2009-2015. Error bars represent standard error.

3.6 Invertebrate Community

A total of 30 taxa were identified at the family level or above over the study period September 2009 to December 2015, but only 19-25 of these were present in each year. The impact of larvicide on the overall invertebrate community structure was assessed in two ways:

(i) ANOVA analysis of taxa richness data; and
(ii) multivariate analysis of community assemblage.

The sections below provide these results for both S-methoprene and *Bti* larvicide applications. References to number of taxa or taxa richness described in this section include the number of macroinvertebrate families plus the number of zooplankton taxa at the level of Copepoda or Cladocera.

3.6.1 Response to S-methoprene

Taxa richness

The number of taxa present in samples ranged from three to sixteen, and variation between application dates was more notable than variation between sample types (Figure 18). Pre-treatment, Post-treatment and control samples had very similar overall mean taxa richness (9.5, 9.5 and 9.6 respectively). Not surprisingly, there was no significant difference between the three sample types ($F_{2,6} = 0.66$, $P = 0.550$; pairwise comparisons $P > 0.333$). Larvicide application date had a highly significant effect on richness for all sample types ($F_{18,36} = 6.66$, $P < 0.01$), and pairwise comparisons showed differences both within and between years, as evident in Figure 18. These results do not indicate any adverse effect of S-methoprene on taxa richness.

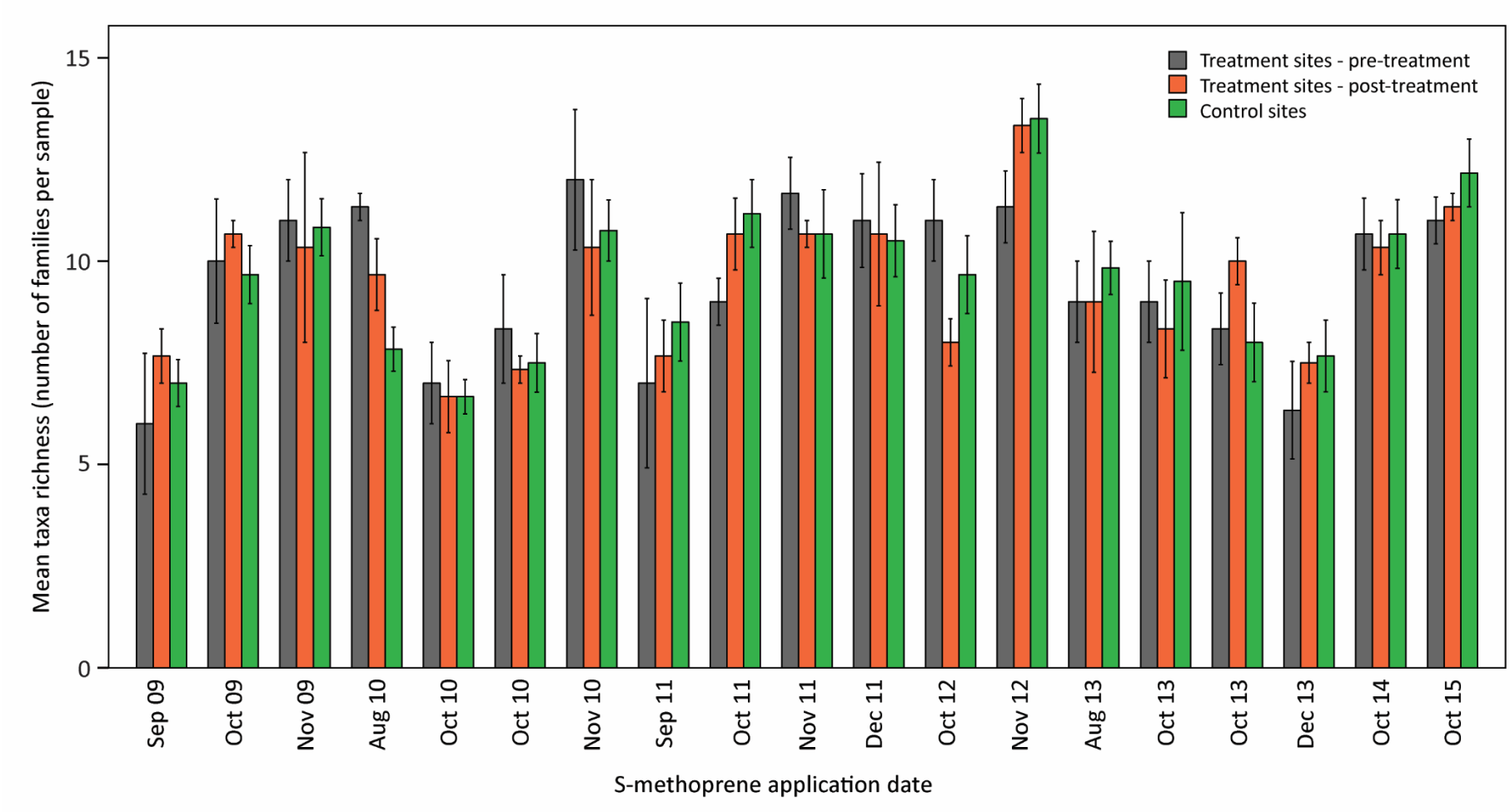

Figure 18. Mean taxa richness in pre- and post-treatment samples from treatment sites and in control site samples for each application of S-methoprene in the Vasse-Wonnerup Wetlands 2009-2015. Error bars represent standard error.

Community Assemblage

Analysis of similarity (ANOSIM) did not indicate any effect of S-methoprene on invertebrate community assemblage, with a very low overall *R* value of -0.036 (*P* = 0.829) indicating a very high level of similarity. Pairwise tests also indicated no significant differences between each of the three sample types:

- pre-treatment and post-treatment samples: *R* = -0.274, *P* = 0.495
- pre-treatment and control samples: *R* = 0.031, *P* = 0.257
- post-treatment and control samples: *R* = -0.003, *P* = 1.0

In contrast, assemblage did differ significantly between application dates (*R* = 0.396, *P* = 0.001). The MDS plots in Figure 19 reflects these results: when data are displayed as the three sample types (Figure 19a), their distribution is indiscriminate; however when data are displayed as application number within each year (Figure 19b), we see greater similarity within these groupings. It is not practical to display data grouped for each S-methoprene application because with 19 applications is too many for meaningful visual display. Application number within each year is considered a reasonable reflection of environmental conditions through each control season.

As there was a high level of similarity between sample types, SIMPER analysis to investigate the taxa contributing to dissimilarity were not necessary.

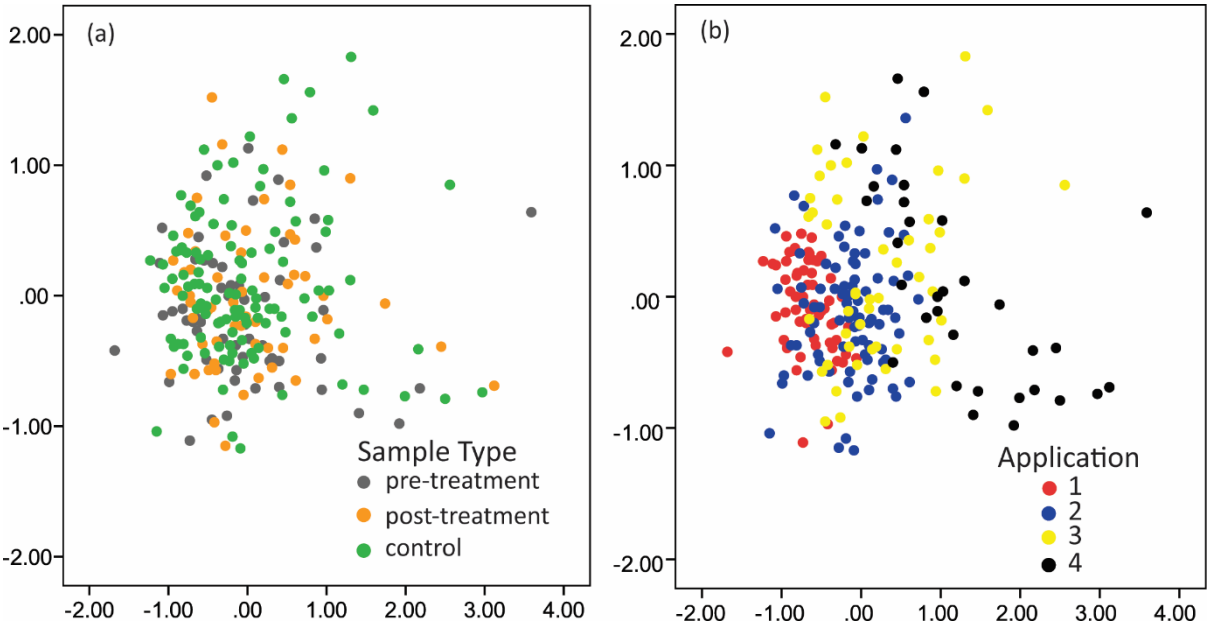

Figure 19. MDS plots of aquatic invertebrate abundance, showing data are sample types (a) and S-methoprene application number, within each year (b). Refer to Table 2 for dates of application in each year.

3.6.2 Response to *Bti*

Taxa richness

There was no evidence for any effect of *Bti* application on taxa richness. Richness in post-treatment samples was lower than pre-treatment samples and control samples following only one of three *Bti* applications (Figure 20). Richness did not differ significantly between the three sample types ($F_{2,6}$ = 0.42, P = 0.678; pairwise comparisons P > 0.428) and overall mean values were similar (pre-treatment: 10.6, post-treatment: 9.9, control: 10.0). Richness did not vary significantly between application dates ($F_{2,4}$ = 3.47, P = 0.065).

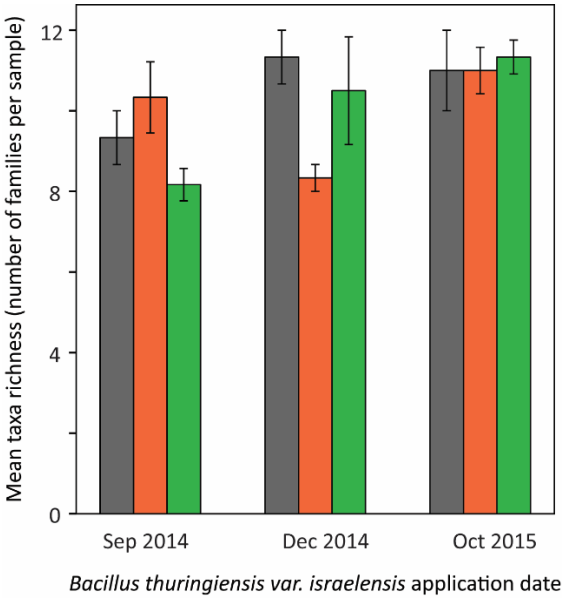

Figure 20. Mean abundance of zooplankton in pre- and post-treatment samples from treatment sites and in control site samples for each application of *Bacillus thuringiensis var. israelensis* in the Vasse-Wonnerup Wetlands 2009-2015. Error bars represent standard error.

Community assemblage

Analysis of similarity (ANOSIM) did not indicate any effect of *Bti* on invertebrate community assemblage, with a very low overall *R* value of 0.059 (*P* = 0.275) indicating a very high level of similarity. Pairwise tests also indicated no significant differences between each of the three sample types:

- pre-treatment and post-treatment samples: *R* = -0.086, *P* = 0.668
- pre-treatment and control samples: *R* = 0.181, *P* = 0.087 (although not significant, this does indicate these sample types were more similar than other pairs.
- post-treatment and control samples: *R* = -0.01, *P* = 0.517

In contrast, assemblage did differ significantly between application dates (*R* = 0.396, *P* = 0.001). The MDS plots in Figure 21 reflects these results: when data are displayed as the three sample types (Figure 21a), their distribution is indiscriminate; however when data are displayed as application dates (Figure 21b), we see distinct grouping of similarity within each application. Pairwise tests for application indicated that assemblage differed significantly between all application dates (*P* < 0.02).

As there was a high level of similarity between sample types, SIMPER analysis to investigate the taxa contributing to dissimilarity were not necessary.

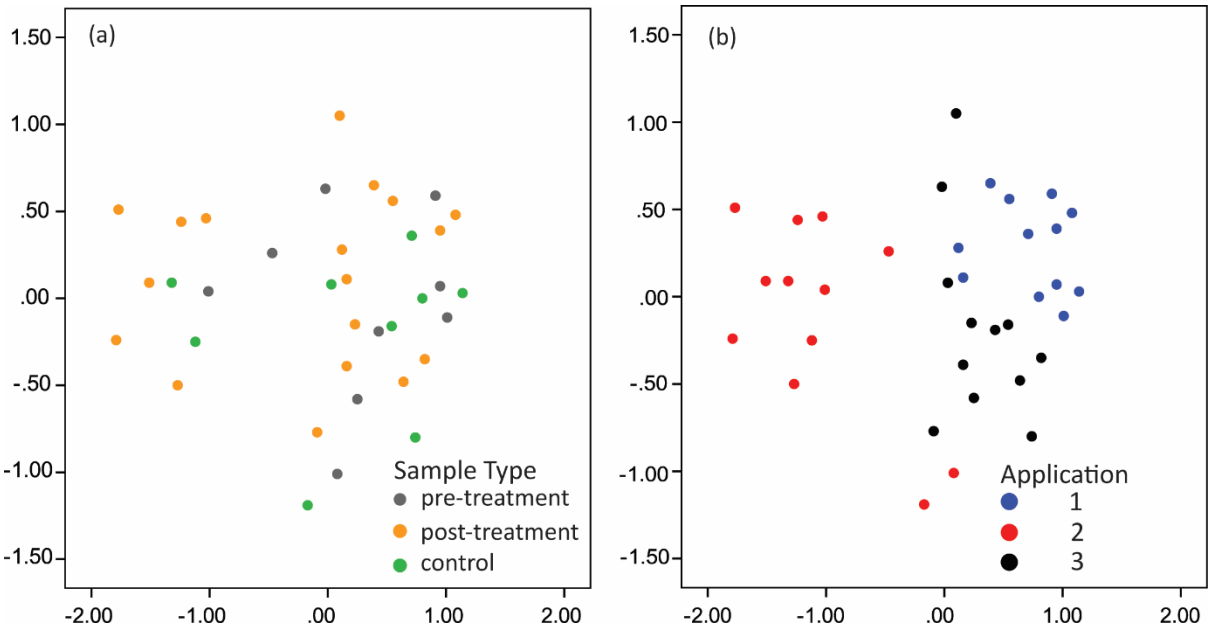

Figure 21. MDS plots of aquatic invertebrate abundance, showing data are sample types (a) and Bti application dates (b). Application numbers 1, 2 and 3 correspond to Bti applications in September 2014, December 2014 and October 2015.

3.7 Hand treatment and fogging

The effect of hand treatment in January 2012 of an area of treatment site T1 was assessed using data from sites T1 and nearby C1. This did not affect invertebrate abundance, with post-treatment abundance higher than or similar to pre-treatment abundance for all main taxa groups (Figure 22). While Figure 22 does show lower Cyprididae (excluding *Mytilocypris*) abundance in the post-treatment sample, results for both samples were quite low (16, 5 respectively) and this is not sufficient to indicate an effect, particularly with a sample size of one. Furthermore, similar abundance was found in the post-treatment sample compared to the control site.

Fogging was undertaken in residential areas on 28 October 2013 in proximity of sites T1 and C1 (Figure 1). Unlike larvicide application, fogging was not undertaken within wetlands, however there is some potential for indirect effects from spray drift and therefore pre-and post-treatment sampling was completed in nearby wetlands. Abundance of most invertebrate groups was similar or higher in post-treatment samples than pre-treatment samples (Figure 23). Sampling showed lower abundance of Chironomidae (midge larvae) in both sites following fogging (Figure 23). It is difficult to attribute this to indirect effects of fogging due to the very low sample size and the highly variable nature of abundance data generally. It is also important to note that subsequent sampling in these sites on 17 December 2013 found extremely high abundance of Chironomidae (T1 = 75000; C1 = 10000). Abundance of ostracods (*Mytolocypris* sp. and other Cyprididae) in site C1 was lower in post-treatment fogging samples (Figure 23) but, again, lack of replication limits robust analysis. In addition, post-treatment abundance was also lower than pre-treatment abundance in other control sites at this time. These sites are a considerable distance from fogging activity and unlikely to be affected, suggesting that other environmental factors contributed to lower abundance.

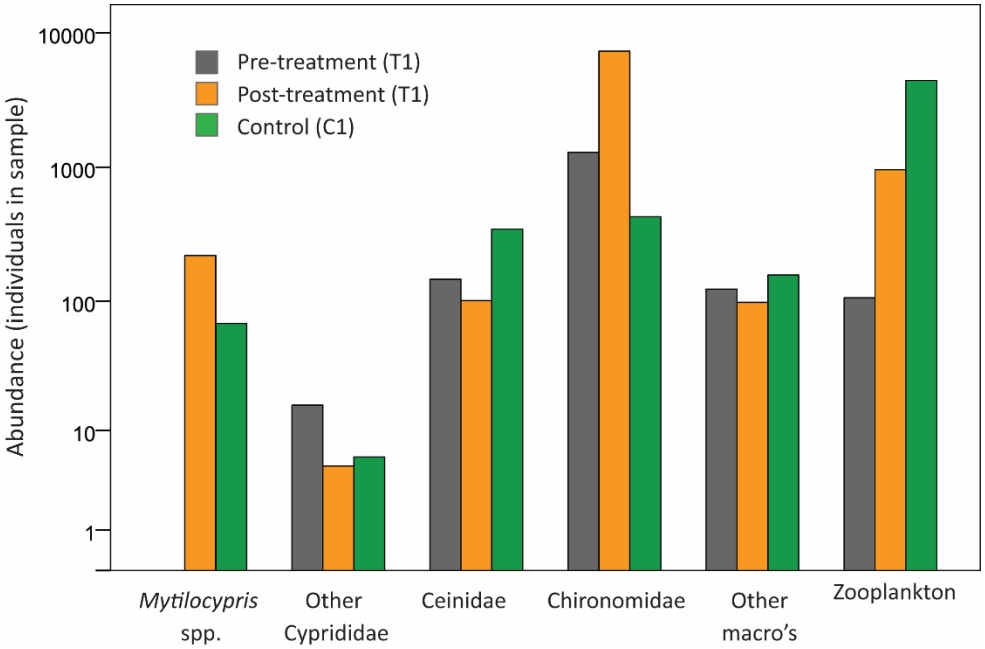

Figure 22. Abundance of major invertebrate groups in sites T1 and C1 during the S-Methoprene hand treatment of T1 in January 2012.

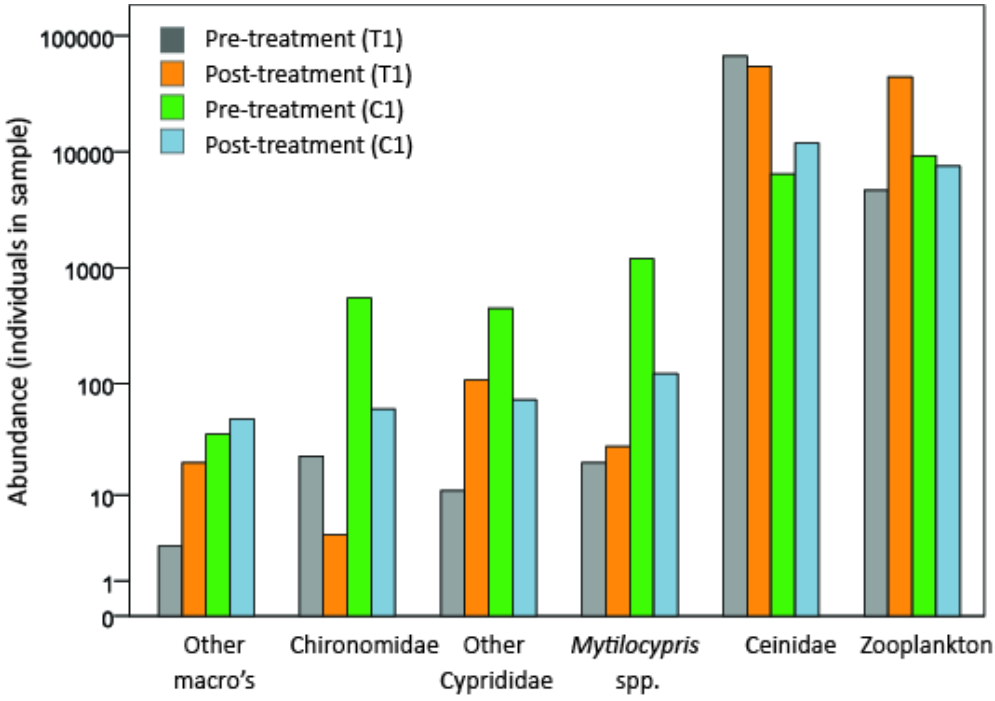

Figure 23. Abundance of major invertebrate groups in sites T1 and C1 during the adulticide fogging treatment in October 2013.

3.8 Larvicide efficacy

3.8.1 S-Methoprene

Data for post-treatment emergence tests was available for most S-Methoprene treatments over the study period (Table 2), however some treatments were not tested in 2011 and 12. Between 3 and 30 individual larvae were collected for each test, but most samples were greater than 10 (mean number collected = 12). Up to seven sites were sampled for each treatment, including locations within the Vasse-Wonnerup wetlands in nearby parts of Busselton. Data was available for 72 samples over the study period, all of which have been included in this analysis. Efficacy of S-methoprene as indicated by emergence tests was generally good (Figure 24), with an overall mean of 84% of collected larvae non-emergent. Mean values were also consistently high (77% and above) for each treatment. Only four samples had emergence of 50% or less.

Although S-Methoprene does not act directly on mosquito larvae populations within treated wetlands, it was considered interesting to test whether its long term use had any overall effect on larvae abundance. Although overall mean was higher in control samples (36) than in pre-treatment (20) and post-treatment samples (19), Culicidae abundance varied greatly (Figure 25) and ANOVA did not show any significant difference between sample types ($F_{2,6} = 0.30$, $P = 0.754$). As for most macroinvertebrate taxa tested, application date was again a significant factor for Culicidae abundance ($F_{18,36} = 10.5$, $P < 0.001$).

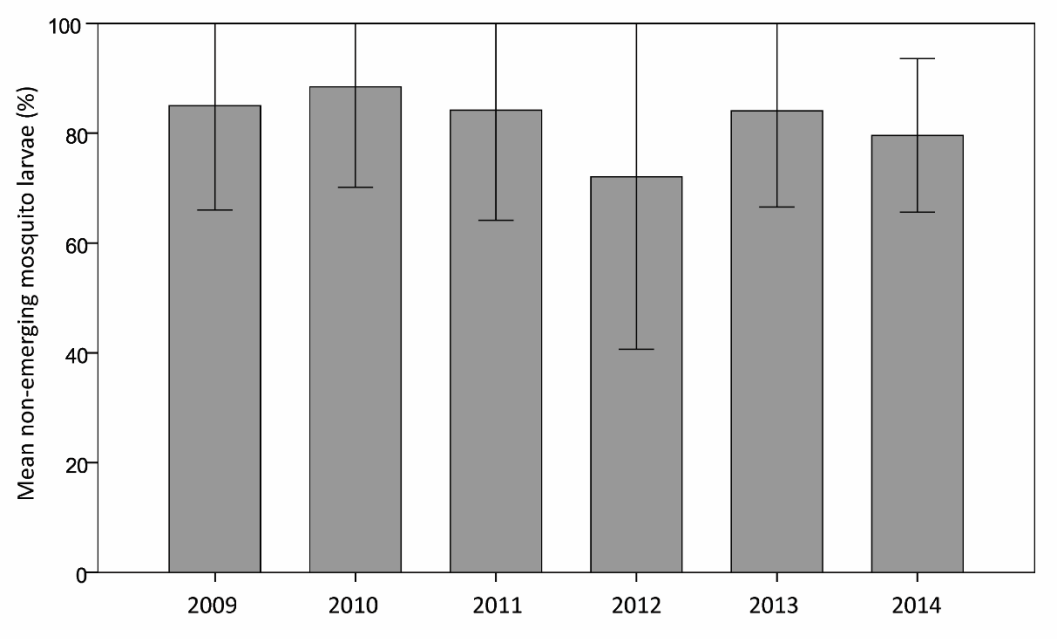

Figure 24. Mean efficacy of S-Methoprene applications in the Busselton wetlands for each year, as indicated by post-treatment mosquito larvae emergence tests. Error bars are standard deviations.

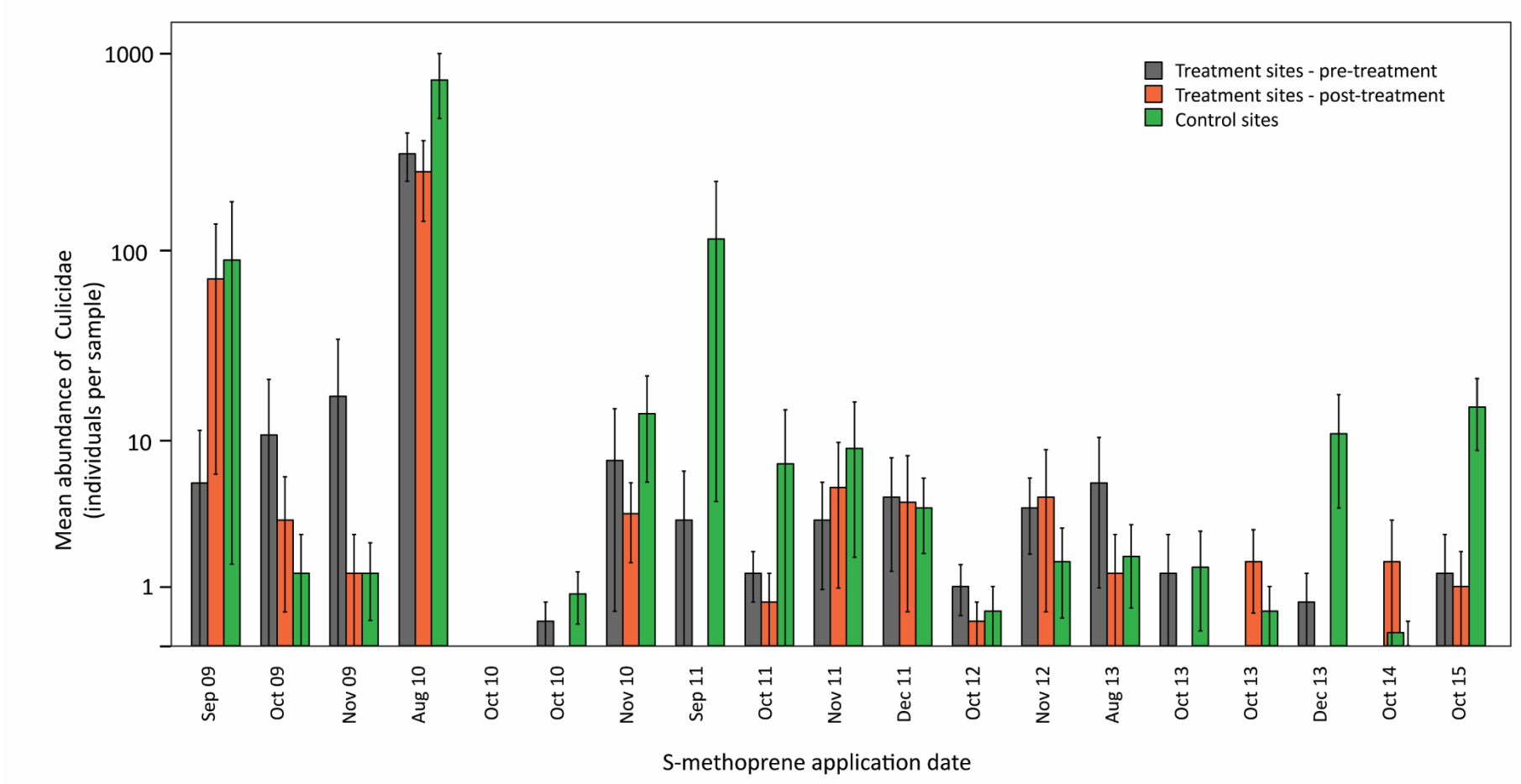

Figure 25. Mean Culicidae abundance in pre- and post-treatment samples from treatment sites and in control site samples for each application of S-methoprene in the Vasse-Wonnerup Wetlands 2009-2015. Error bars represent standard error.

3.8.2 *Bacillus thuringiensis var. israelensis*

Three treatments have been completed and pre- and post-treatment data includes replicate (3-5) dip samples from 6 sites in the Vasse-Wonnerup wetlands, though not all sites were sampled each time. Significantly lower abundance of mosquito larvae was evident following each *Bti* treatment (Figure 26; $F_{1,2}$=12.81, P = 0.005). Post-treatment dip samples had very low abundance, with most samples containing zero larvae (mean abundance = 0.9). These results indicate a mean reduction of 95% of mosquito larvae in response to *Bti* treatment, suggesting high efficacy of this agent. Because *Bti* acts directly on larvae, there is also potential to use non-target invertebrate sample data to assess efficacy of these applications. However Culicidae abundance was too low in these wetlands to enable such an assessment.

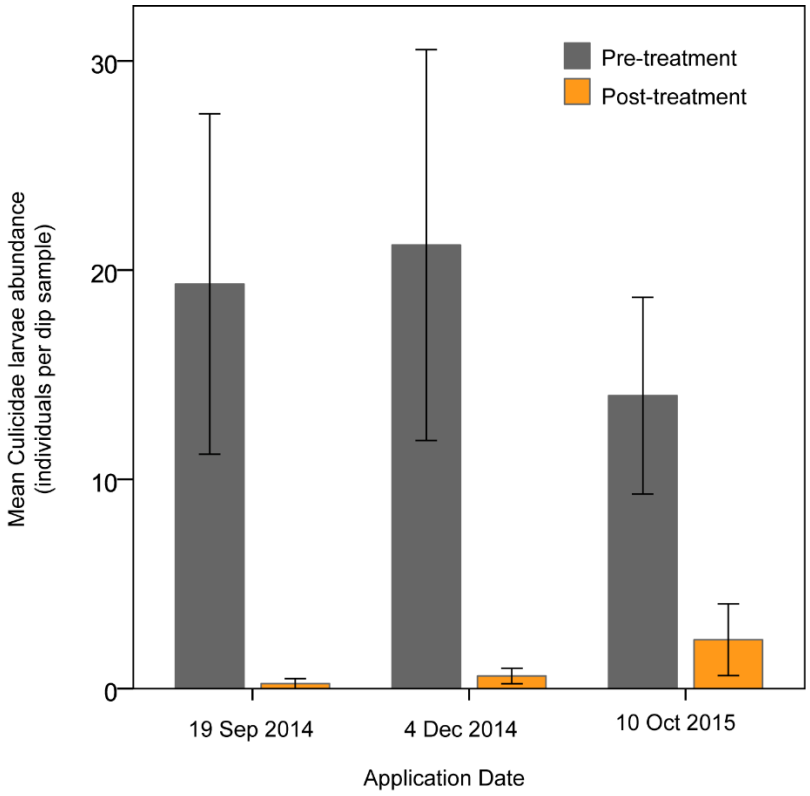

Figure 26. Mean abundance of Culicidae larvae in pre- and post-treatment dip samples in the Vasse-Wonnerup Wetlands for each application of *Bacillus thuringiensis var. israelensis*. Error bars represent standard error.

4 Discussion

Results of this sampling program did not indicate any negative impact of the use of S-Methoprene and *Bti* larvicide on aquatic invertebrate communities. Given this outcome, any indirect impact on waterbirds through alterations to food availability are considered highly unlikely. Abundance data did not show any consistent reduction in response to larvicide tests for the indicator taxa, other macroinvertebrate groups or richness and statistical testing proved that there was no difference between pre-treatment, post-treatment and control samples. In the case of S-methoprene, the availability of long term (seven years) of data for this study and the high level of non-significance found between sample types provides a high level of confidence in these findings. For *Bti*, only three applications have been completed and additional assessment would increase confidence in the results, particularly given the high variation between sites. The date of application was a significant factor for abundance of all invertebrate groups and for taxa richness during S-Methoprene treatments, and for most groups during *Bti* treatments (not for zooplankton or amphipods). Multivariate analysis also indicated a significant effect of timing of application for both larvicide agents. Given the lack of difference between sample types, this doubtless reflecting natural seasonal changes in community structure in the sampled wetlands. Patterns of invertebrate succession in wetlands due to changes in physical and chemical factors and due to biological interactions are common (Lake et al. 1989; Boulton et al. 2014). Seasonal changes in food availability, different tolerance levels for temperature, salinity and drying, and changing predatory-prey relationships are some examples of natural processes underlying seasonal changes in invertebrate communities. It appears clear that invertebrate abundance in these wetlands vary independently of mosquito control activities.

In addition to seasonal changes in invertebrate communities, which contributes to variation within each site, variation between sites is influenced by the particular physical, chemical and biological characteristics of the sampled wetlands. Although similar in the sense that they are all shallow open wetlands within a seasonally inundated samphire marsh, each has a unique characteristics of aquatic vegetation, seasonal depth variation, filamentous algal growth, and temperature sand salinity regimes; all of which change over time in response to climatic conditions. These factors have important influence on habitat quality and food availability with consequences for the aquatic fauna, and are likely to prevail over larvicide use as dominant factors structuring aquatic invertebrate community. Aquatic invertebrate abundance is notoriously variable, even between sites with very similar ecological conditions (Lagadic et al. 2016) and the use of replicate sampling sites in this study was important to account for this.

The findings of this sampling program are consistent with other studies which have found negligible effects on non-target aquatic invertebrates of S-methoprene at rates required for effective mosquito control (eg. Pinkney *et al*, 2000; Russel *et al*, 2009), and show additionally that impacts are unlikely

even after long term use (seven years). This outcome is important because there are few long-term field investigations of impacts of S-methoprene on non-target organisms. While methoprene is toxic to a range of invertebrates, lethal doses are highly variable and the very low rate required for control of mosquito larvae makes its application for this purpose very specific (Stark, 2005a). Toxic effects of methoprene on non-target species are expected at levels substantially higher than those which would be produced by application for mosquito larvae control (Glare and O'Callaghan, 1999; Stark, 2005a). While S-methoprene is an effective agent for mosquito control, there is potential for resistance to develop in mosquito populations (Baldacchino et al. 2015), and *Bti* may be preferred for future control. However, the high efficacy of S-Methoprene in the City of Busselton indicates that resistance is not an issue at present.

There have been many field studies of the potential impacts of *Bti* on wetland invertebrate communities, and the absence of negative impacts shown in this study support the findings of most other research. Although Notonectidae abundance was lower in post-treatment samples following the December 2014 application, *Bti* has shown no toxicity to this group in laboratory trials (Ser et al. 2015). With only three applications over two years, the data in the present study is considered limited. Moreover, the key non-target group potentially impacted by *Bti*, i.e. Chironomidae, would be more effectively collected using benthic sampling methods rather than water column sweeps. Decreased chironomid abundance and decreased overall invertebrate richness and biomass was found in one short-term (2 years) field study (Hershey et al.1998), but many long-term studies have shown no effects, irrespective of the frequency of treatments (Lagadic et al. 2016). These studies have also found an overriding influence of environmental factors on community dynamics. Some research in the Camargue, in France, where 30 to 50 treatments are applied each year, suggest that adverse impacts on chironomids can have indirect effects on predatory Odonata (dragonflies) and birds (Poulin et al. 2010; Jakob and Poulin 2016), however there has been criticism of the methods used (Lagadic et al. 2014). *Bti* is being used increasingly worldwide, owing to low toxicity to non-target organisms and the potential for resistance to chemical agents (including S-Methoprene) (Baldacchino et al 2015), however concern remains despite much evidence that it does not impact non-target organisms. *Bti* is generally highly effective at the recommended application rates (90%, Lagadic et al. 2016), and this was apparent in efficacy sampling for its use in this program to date. It should be noted that higher rates sometimes suggested for extended control can adversely affect microbial organisms (Duguma et al. 2015), with consequences for community dynamics.

Fogging of residential areas had no impact on abundance of most invertebrate groups. Reduced abundance of chironomids in two adjacent site and ostracods in one adjacent site do not provide clear evidence of impacts due to a lack of replication over space or time, and additional replicate sampling should be used in future assessment of impacts. Measurement of pyrethroid concentrations in the water column may be also be useful. Fogging is not used directly over wetlands, and the requirement

for light wind conditions (maximum 8 knots) for this activity limits the potential contamination of wetlands with the pyrethroid insecticides used. Current research indicates that while pyrethroids are highly toxic to a range of aquatic invertebrates, impacts under field conditions are mediated by low solubility and where impacts have been observed (most commonly associated with agricultural applications), populations usually recover within weeks (Antwi and Reddy 2015). Short-term impacts of pyrethroids on Chironomidae have been found, but recovery is rapid owing to a very short life cycle and high recolonisation potential (Conrad et al. 1999). The most vulnerable invertebrate groups to impacts are insects, particularly Ephemeroptera, Plecoptera, Tricoptera and Odonata (Antwi and Reddy 2015), and there are no available studies on potential impacts on Ostracoda. Application rates used by the City of Busselton have potential to result in concentrations of only 0.1 - 1.0 % of toxic levels if used directly over wetlands (Paice 2011a). Thus, use of adulticide in adjacent areas is unlikely to impact non-target organisms when applied under light wind conditions and at the rate presently used.

The aim of the sampling program was to determine any negative impacts of mosquito larvicide on non-target aquatic invertebrates, and thereby assess the potential impact on waterbird food resources. As the data does not suggest any influence of larvicide on aquatic invertebrates, consequent impact on waterbirds through alterations to food availability is highly unlikely. Further, larvicide spraying is generally restricted to peripheral areas of the wetlands, and as these areas dry out, waterbirds move to open water areas which are excluded from larvicide treatment and support high densities of aquatic invertebrates (Chambers et al, 2010). The low level of risk to non-target aquatic invertebrates from larvicide use is due to the very low rates required for mosquito control. It is therefore critical that appropriate application rates are used, particularly in environmentally sensitive areas. Negligible impacts on non-target organisms, together with high effectiveness, indicate appropriate use of larvicide by the City of Busselton.

5 References

Antwi, F. B. and Reddy, G. V. P. (2015) Toxicological effects of pyrethroids on non-target aquatic insects. *Environmental Toxicology and Pharmacology* **40**: 915-923.

Australian Wetlands Database (2010)
http://www.environment.gov.au/water/topics/wetlands/database/

Baldacchino, F., Caputo, B., Chandre, F., Drago, A., della Torre, A., Montarsi, F. and Rizzoli, A. (2015). Control methods against invasive *Aedes* mosquitoes in Europe: a review. *Pest Management Science* **71**: 1471-1485.

Boulton, A. J., Brock, M. A., Robson, B. J., Ryder, D. S., Chambers, J. M. and Davis, J. A. (2014). *Australian Freshwater Ecology*. Wiley Blackwell, U.K.

Chambers, J.M., Clarke, A., Strehlow, K. and Paling, E.I. (2010) *Snapshot Survey of the Distribution and Abundance of the Macrophytes, Macroalgae, Phytoplankton and Macroinvertebrates of the Vasse-Wonnerup Lagoons November, 2009 and February, 2010.* Marine and Freshwater Research Laboratory, Environmental Science (Report No. MAFRA 10/4), Murdoch University, Murdoch.

Chessman, B. C., Trayler, K. M. And Davis, J. A. (2002). Family- and species-level biotic indices for macroinvertebrates of wetlands on the Swan Coastal Plain. *Marine and Freshwater Research* **53:** 919-930.

Conrad, A.U., Fleming, R.J. and Crane, M., (1999). Laboratory and field response of *Chironomus riparius* to a pyrethroid insecticide. *Water Research* **33**: 1603–1610.

Davis, J. and Christidis, F. (1997). *A Guide to Wetland Invertebrates of Southwestern Australia.* Western Australian Museum, Perth.

Duguma, D., Hall, M. W., Rugman-Jones, P., Stouthamer, R., Neufeld, J. D. and Walton, W. E. (2015). Microbial communities and nutrient dynamics in experimental microcosms are altered after the application of a high dose of *Bti*. *Journal of Applied Ecology* **52**: 763-773.

Glare, T.R. and O'Callaghan, M. (1999). Environmental and health impacts of the insect juvenile hormone analogue, S-methoprene. Report for the Ministry of Health, New Zealand.

Hershey, A.E., Lima, A.R., Niemi, G.J. and Regal, R.R. (1998). Effects of *Bacillus thuringiensis israelensis* (*Bti*) and methoprene on non-target macroinvertebrates in Minnesota wetlands. *Ecological Applications*. **8**: 41–60.

Jakob, C. and Poulin, B. (2016). Indirect effects of mosquito control using *Bti* on dragonflies and damselflies (Odonata) in the Camarge. *Insect Conservation and Diversity.*

Lagadic, L., Roucaute, M. and Caquet, T. (2014) *Bti* sprays do not adversely affect non-target aquatic invertebrates in French Atlantic coastal wetlands. *Journal of Applied Ecology* **51**: 102-113.

Lagadic, L., Shaefer, R. B., Roucaute, M., Chouin, S., de Maupeou, J., Duchet, C., et al. (2016). No association between the use of *Bti* for mosquito control and the dynamics of non-target aquatic invertebrates in French coastal and continental wetlands. *Science of the Total Environment* **553**: 486-494.

Lake P.S., Bayly, I. A. E. and Morton, D. W. (1989) The phenology of a temporary pond in western Victoria, Australia, with special reference to invertebrate succession. *Archiv für Hydrobiologie* **115**: 171-202.

Lane, J. A., Clarke, A. G., Pearson, G. B. and Winchcombe, Y. C. (2007). *Waterbirds of the Vasse-Wonnerup Wetlands in 1998-2000, including Ramsar Status and comparisons with earlier data*.

Paice, R. (2010) *Potential of aerial larvicide applications to impact on aquatic invertebrate prey species for waterbirds in the Vasse-Wonnerup Wetland System*. Report to the Shire of Busselton, Western Australia.

Paice, R. (2011a) Environmental Management Plan for Mosquito Control Activities in the Vasse-Wonnerup Wetland System. Report to the Shire of Busselton, Western Australia.

Paice, R. (2011b) Impact of aerial larvicide on aquatic invertebrates in the Vasse-Wonnerup wetland system: Results of 2010 monitoring. Report to the Shire of Busselton, Western Australia.

Paice, R. (2012) Impact of aerial larvicide on aquatic invertebrates in the Vasse-Wonnerup wetland system: Results of 2011-12 monitoring. Report to the City of Busselton, Western Australia.

Paice, R. (2013) Impact of aerial larvicide for mosquito control on aquatic invertebrates in the Vasse-Wonnerup Wetland System: results of 2012 sampling. Report to the City of Busselton, Western Australia.

Paice, R. (2014) Impact of aerial larvicide for mosquito control on aquatic invertebrates in the Vasse-Wonnerup Wetland System: results of 2013 sampling. Report to the City of Busselton, Western Australia.

Paice, R. (2015) Impact of aerial larvicide for mosquito control on aquatic invertebrates in the Vasse-Wonnerup Wetland System: results of 2014 sampling. Report to the City of Busselton, Western Australia.

Paice, R. (2016) Impact of aerial larvicide for mosquito control on aquatic invertebrates in the Vasse-Wonnerup Wetland System: results of 2014 sampling. Report to the City of Busselton, Western Australia.

Pinkney, A. E., McGowan, P. C., Murphy, D. R., Lowe, T. P., Sparling, D. W. and Ferrington. L. C. (2000). Effects of the mosquito larvicides temephos and methoprene on insect populations in experimental ponds. *Environmental Toxicology and Chemistry* **19**: 678-684.

Poulin, B., Lefebvre, G. and Paz, L. (2010). Red flag for green spray: adverse trophic effects of *Bti* on breeding birds. *Journal of Applied Ecology* **47**: 884-889.

Russell, T. L., Kay, B. H. and Skilleter, G. A. (2009) Environmental effects of mosquito insecticides on saltmarsh invertebrate fauna. *Aquatic Biology* **6**: 77-90.

Stark, J. D. (2005a) A Review and Update of the Report “Environmental and health impacts of *Bacillus thuringiensis israelensis*” 1999 by Travis R. Glare and Maureen O'Callaghan. Report for New Zealand Ministry of Health, Wellington.